

Proteomic profiling of UV damage repair patches uncovers histone chaperones with central functions in chromatin repair

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The current manuscript presents a development of the IPOND technique aimed at capturing the proteome at UVC damage repair sites (IPOND-Repair). This is an interesting new technique that expands the arsenal of methods to study DNA repair in the context of chromatin, and could be further applied to explore the proteome at sites of diverse DNA lesions that require DNA synthesis for their resolution. Using IPOND-R, the authors identify ~250 proteins enriched at UVC damage sites (one-hour post-irradiation). These include several histone chaperones (DNAJC9, MCM2, CAF1, FACT...), nucleosome remodelling enzymes and histone modifiers. The work then focuses on the function of DNAJC9 and MCM2 at UVC damage sites. Chromatin is dynamically remodelled at UVC damage sites, including the transient displacement and recovery of 'old' histones, and the deposition of 'new' histones. A full picture of which histone chaperones are involved in this remodelling of chromatin is missing, and this work represents an important advance. Here, the authors show that DNAJC9 is required for incorporation of new histones H3 (H3.1 and H3.3 variants) at UVC damage sites (as well as their genome-wide incorporation); and that DNAJC9 and MCM2 similarly function in the recovery of old histone H3.3 levels at UVC repair sites. Together, the work implicates DNAJC9 and MCM2 in the maintenance of chromatin structure at UVC damage sites, without affecting the actual repair of UV lesions.

The main point that would greatly strengthen the manuscript is a deeper mechanistic understanding of how DNAJC9 and MCM2 function at sites of DNA damage. First, the establishment of a functional rescue system will be key to unequivocally assign the function of DNAJC9 and MCM2 during chromatin remodelling at UVC damage sites. Second, as also pointed out by the authors, based on the current literature a number of mechanisms can be invoked to explain how DNAJC9 and MCM2 accumulate at sites of DNA damage to promote histone incorporation. These could be direct binding to histone H3-H4 (histones evicted from chromatin could become 'visible' to these chaperones?); interaction of MCM2 with other CMG components; regulation of 'damaged' chromatin binding by the J domain of DNAJC9. Importantly, point mutants that disrupt these interactions have all been described. The contribution of these mechanisms could be tested in a rescue system, and would transform our understanding of how DNAJC9 and MCM2 function at DNA repair sites.

Other points:

Line 116: Figure S1E only shows XPB recruitment, not 'UV damage dependent factors', so the text should be amended accordingly.

Fig. 2D is not very informative, particularly the 'reference number' for the gene ontology terms. Can this panel be reshaped? How do the authors explain the difference in proteins retrieved by aniFOUND as compared to IPOND-R (Fig. 3A)? adding a short explanation in the text would be helpful for readers to compare the two techniques. And how is the overlap between IPOND-R and IPOND (untreated conditions)?

In Figure 4, the pattern of H3.1 incorporation into chromatin is used to define whether cells are in S phase or not. However, given that loss of DNAJC9 decreases new H3.1-SNAP incorporation and the percentage of S-phase cells (Fig. S5G-I), how reliable is the observed H3.1 pattern? Can the authors test whether DNAJC9-depleted cells still consistently show the expected 'S-phase' (early, mid, late) patterns for H3.1-SNAP, p150 and/or EdU? This would validate the use of H3.1-SNAP patterns as a marker of S-phase in Fig. 4.

Fig. 4H: Given that DNACJ9 and HIRA depletion leads to lower H3.3-SNAP incorporation, the H3.3-SNAP images seem not very representative. Has the contrast been changed differently between samples to highlight the decrease in H3.3-SNAP accumulation specifically at UVC sites? if so, this should be stated in the legend.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

The paper by Plessier et al, describes a new proteomic method to isolate factors accumulating on newly synthesized DNA after UV DNA damage, named IPOND-R. After validation of the method, the authors focus on analyzing the effect of depletion of DNAJC9 on the dynamics of new and old histones at DNA damage sites. The authors find that DNAJC9 controls the dynamics of both histone populations, but does not affect DNA damage repair per se (i.e. recruitment of repair proteins, or survival after DNA damage). The authors conclude by suggesting an interplay between MCM2 and DNAJC9 to control chromatin dynamics at UV damage sites.

The paper contains high quality analyses of the effect of DNAJC9 depletion, and also provides a well validated new approach to study the proteome of DNA damage repair sites. In general, the findings are interesting and may attract attention from several research communities involved in DNA repair and chromatin biology. However, there are few concerns that need to be addressed before publication.

The most critical issue is that it is not clear that the effects described are direct and related to DNA damage repair, rather than indirect effects from the cells going through S-phase without critical histone chaperones. DNAJC9, MCM2 as well as CAF-1 and HIRA are critical histone chaperones and they will alter chromatin dynamics independently of DNA damage, as previously shown. Because the authors do not see an effect of DNAJC9 on DNA repair or survival after damage, it could be that the authors are simply measuring changes in chromatin dynamics that are not dependent on DNA damage, but related to global chromatin alterations. This is due in part to the experimental setup of the assay: the use of siRNA and the conditions used to inhibit DNA replication. Controls should be added to address this concern.

1) The authors run many important controls to validate the IPOND-R approach to minimize the contribution of DNA replication in the dataset. However, treatment of cells with HU and Ara-C to inhibit replicative synthesis also stresses these cells significantly. I wonder if the authors could use synchronization of the cells in G1/S by Palbociclib (which is known to not induce stress) to conclusively demonstrate the power of the method. Running IPOND-R and the follow up (key) experiments in Palbociclib arrested cells will provide important confirmation that DNA replication and S-phase mechanisms are not at play in the chromatin dynamics presented here.

2) As the authors deplete their proteins with siRNA, the cells are cycling in the absence of the target proteins for at least one full cell cycle. As the authors are targeting critical histone chaperones (DNAJC9, MCM2, CAF-1 and HIRA), it is expected that chromatin is already affected at the time of DNA damage induction. The authors should find ways to circumvent this experimental limitation (i.e. use rapid degron-based degradation or test their key findings in G1 arrested cells).

3) In line 228-229 it is stated that the quantification of Figure 4B focuses on non S-phase cells. It is not clear to this reviewer how one can reliably distinguish S and non S-phase cells from new H3.1 distribution (I can only imagine a difference between early and late S-phase). I expect a difference could be in the total amount of new H3.1 (should be significantly higher in S-phase cells), but not clear how the distribution can alone explain S vs non-S phase cells. At any rate, the authors should include a quantification for S-phase cells to test if there is a difference. Could the authors use EdU to distinguish various populations otherwise? Or synchronize with Palbociclib as suggested above?

4) The involvement of MCM2 is also unusual, without evoking the passage of DNA replication forks. The authors do not really mention this, but this needs to be addressed/discussed. How can they envision MCM2 working at these sites, or together with DNAJC9, outside of DNA replication? Hypotheses need to be tested to clarify their data, which is very preliminary in the current form.

5) In Figure 1D, the authors should probe for DNA replication proteins to show that they are not pulled down (e.g. Cdc45, GINS, or fork protection complex components).

6) The authors should also show the overlap between IPOND-R and previously reported IPOND hits (in Figure 2), to show how many of the fork proteins are present in the data.

There is an instance of "data not shown" in the discussion (lines 339-340). These data should be shown, or the statement needs to be removed.

Reviewer #4

(Remarks to the Author)

The study investigates chromatin dynamics during UV damage repair, focusing on identifying proteins that regulate histone deposition at damaged sites. The authors modified the IPOND proteomic method, naming it IPOND-R, to analyze protein dynamics specific to repair sites. Through this approach, they identified DNAJC9 and MCM2, which had not previously been linked to UV-induced damage. DNAJC9 is suggested to facilitate the deposition of new histones and recovery of parental

histones, while MCM2 is proposed to collaborate in these processes to maintain chromatin integrity. This finding adds to the understanding of the dynamic regulation of chromatin following DNA damage. However, several issues undermine the study's conclusions. Many results lack appropriate controls, and the experimental descriptions are insufficiently detailed, making the findings difficult to interpret and raising concerns about their reliability. Furthermore, the logical connections between key results are unclear. These shortcomings lead to ambiguous conclusions, and some interpretations appear to extend beyond what the data can support.

Major:

1. Based on the proposed model of chromatin repair (Figure 6F), it appears that DNA breaks are repaired first, followed by histone deposition to restore chromatin structure. However, the authors did not assess when DNA repair is complete. Notably, the persistence of γ H2AX after 24 hours suggests that repair may not be fully resolved, and DNAJC9 is no longer present at the damage sites at this time point. Furthermore, while DNAJC9 is transiently present at UVC damage sites, the authors report no evidence of its involvement in DNA repair. These findings raise important questions about the proposed model and the role of DNAJC9 at DNA damage sites.

To clarify these points and strengthen the model, the authors should provide additional details, including:

- Evidence indicating when DNA repair is complete.
- Whether the loss of DNAJC9 affects transcription recovery near break sites following UVC damage.

Addressing these points will help establish a clearer connection between DNA repair, histone deposition, and the function of DNAJC9 in the context of DNA damage.

2. The authors treated cells with 10 mM HU and 1 mM AraC to inhibit DNA replication, raising several concerns:

- While AraC can inhibit DNA replication, it is also incorporated into repair sites, potentially causing repair defects. This issue is not addressed in the manuscript, undermining the validity of the experimental approach.
- The use of 1 mM AraC is an extremely high concentration for dividing cells, especially since concentrations as low as 40 μ M can be toxic to some post-mitotic cells. The observation that cells accumulate in the G1 phase even 24 hours post-treatment with DNA synthesis inhibitors suggests that the inhibitors themselves may have induced significant cellular stress. Given these concerns, we recommend replacing HU+AraC with other inhibitors, such as G1 arrest inhibitors (eg. Pablociclib), which would allow the cells to arrest in G1 phase and prevent EdU incorporation during DNA replication. This adjustment would enhance the reliability of the approach and reduce potential confounding effects.

3. In Figure 5B, siXPG should prevent UV lesion removal, thereby halting DNA repair. However, the data still show a recovery of old H3.3, which is confusing. If DNA repair is blocked, it would be expected to also impact histone deposition. The authors should clarify how histone deposition occurs independently of DNA repair in this context and provide additional insights into DNAJC9's function.

Minor:

1. To identify proteins that transiently bind to chromatin during repair, the authors compared IPOND-R results at 1-hour and 24-hour post UV time points. However, this approach does not rule out the possibility that cells with the most severe damage at 1 hour post-UV may not survive until the 24-hour time point, thus create bias. To address these concerns, the authors should perform EdU pull-down sequencing to confirm that the EdU-incorporated sites at 24 hours are comparable in abundance and distribution to those at 1 hour post-UV treatment.

2. The analysis in Figures 4 and 5 presents data at 20 minutes, 2 hours, and 8 hours post-UVC exposure without providing any explanation for this shift, despite earlier data focusing on 1 hour post-UVC. This raises questions about whether DNAJC9 remains present at UV damage sites at these time points in the control. To address these issues, the authors should provide a clear rationale for the time-point selection.

3. The IF results for new H3.3-SNAP in siDNAJC9#3 in Figures 4C and 4H display inconsistent deposition patterns (completely absent in one instance and still present in another), which raises concerns about data reliability. The authors should use figures displaying multiple cells instead of single cells to ensure greater consistency and reliability in the presentation of the data.

4. There is no description of how the survival assay was performed. It is unclear whether the UVC dose of 10 J/m² causes 85% cell death within 24 hours, 48 hours, or over the course of a week, which creates confusion and raises questions about the experimental conditions.

In light of these concerns, the paper's conclusions about DNAJC9's role in chromatin repair appear premature and not fully supported by the data presented. A substantial revision addressing these issues would be necessary to strengthen the study's findings and interpretations.

Reviewer #5

(Remarks to the Author)

Plessier et al presents an innovative proteomic approach to capture chromatin in the process of UVC irradiation repair based on the IPOND protocol (IPOND-R). This protocol relies on the complete inhibition of DNA replication such that EdU is only incorporated during UV-damage coupled DNA repair processes. As can be seen in Figure 1B, the inhibition of DNA

replication with HU and Ara-C combined is highly effective. Furthermore, the post UVC repair EdU signal is similar in non S-phase cells when not treated with these inhibitors (Figure 1C). These are important controls since small amounts of DNA replication could significantly skew the interpretation of the resulting proteomic analyses from these experiments. Whilst the proteomic datasets have merit, there should be a more detailed comparison and benchmarking of them compared to classical iPOND experiments. For example, by comparing them to the datasets in Sirbu et al JBC VOL. 288, NO. 44, pp. 31458 –3146 (2013). This would help to highlight the differences to factors identified at the replication fork to those enriched at sites of UV damage repair.

Beyond this, I was not sufficiently convinced by the follow-up studies whereby DNAJC9 was selected as the key factor in this process despite being close to the cut-off for statistical significance in the proteomic datasets. The most convincing aspect of this was that depletion of DNAJC9 causes a loss of new SNAP tagged H3.3/H3.1. In the UV irradiated experiments, the signal of DNAJC9 and MCM2 at irradiated sites was not convincing, no easily observable foci are formed. Furthermore, since this could also be explained by bleed through from the GFP channel into the red channel, and I was not able to find evidence that this has been correctly controlled for, this calls into doubt the validity of conclusions made based on this approach – thus at this stage it is unclear of the significance of many findings. Furthermore, the statistical handling of data is questionable. Whilst microscopy experiments have been performed with three biological replicates, paired t-tests appear to use all data points from these experiments rather than the three means of the biological replicates. This is a very confusing way to present the data and makes it difficult to assess how robust the conclusions made are. For example, are the changes in Figure 5 really strong enough to conclude that DNAJC9 has a role in old histone recovery/histone recycling?

Together, whilst I believe this is a promising story, it is not yet sufficiently developed for publication in Nature Comms.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have answered most of the questions we raised. A couple of minor points need to be clarified in the text.

Fig. 4D: in the H3.3-SNAP panel, what are the authors measuring? Is it the total levels of H3.3-SNAP expression? In siLuc or siDNAJC9 cells (and if it is in the latter, how does that fit with DNAJC9 leading to overall decreased histone incorporation?)

On page 13, the authors state that 'DNAJC9 likely provides new H3-H4 histones to CAF-1 and HIRA chaperones because DNAJC9 is loosely bound to UV-damaged chromatin, as it is mostly solubilized by detergents'. While I agree that DNAJC9 likely acts upstream of HIRA and CAF1, I think that this cannot be inferred by a detergent extraction method. There is literature suggesting this, and I think that this sentence should be rephrased accordingly.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

The authors have done a good job at addressing my concerns, it's unfortunate that they could not perform IPOND-R after treatment with Palbociclib due to technical reasons. Nonetheless, the manuscript has significantly improved and now provides important contributions to the field, that should be published.

Reviewer #4

(Remarks to the Author)

The authors have adequately addressed most comments from the reviewers. I'm still somewhat concerned about whether the histone chaperones at sites of DNA repair at UV lesions is related to stalled replication forks. However, the authors tried to address this concern and the results are consistent with their hypothesis. Thus, I do recommend publication.

Reviewer #5

(Remarks to the Author)

Reviewer #5:

- Please see comments below in red. Some are major concerns which are easily addressed, yet authors have not attempted to address them. Specifically, my main concerns are surrounding the significance of the data presented in Figure 5. I have

detailed steps to address my concerns below, and these should be addressed prior to acceptance for publication.

- In addition to the steps below, I noted Sandra Piquet is missing from the author list in the manuscript file.

Plessier et al presents an innovative proteomic approach to capture chromatin in the process of UVC irradiation repair based on the IPOND protocol (IPOND-R). This protocol relies on the complete inhibition of DNA replication such that EdU is only incorporated during UV-damage coupled DNA repair processes. As can be seen in Figure 1B, the inhibition of DNA replication with HU and Ara-C combined is highly effective. Furthermore, the post UVC repair EdU signal is similar in non S-phase cells when not treated with these inhibitors (Figure 1C). These are important controls since small amounts of DNA replication could significantly skew the interpretation of the resulting proteomic analyses from these experiments. Whilst the proteomic datasets have merit, there should be a more detailed comparison and benchmarking of them compared to classical IPOND experiments. For example, by comparing them to the datasets in Sirbu et al JBC VOL. 288, NO. 44, pp. 31458–3146 (2013). This would help to highlight the differences to factors identified at the replication fork to those enriched at sites of UV damage repair.

As suggested, we have compared the IPOND-R mass spectrometry dataset (1 h-specific hits in U2OS cells) to the proteins identified on nascent replicated strands in undamaged conditions by IPOND-mass spectrometry (593 proteins across 4 human cell lines HEK293T, HCT-116, HeLa and RPE-1 (PMID: 31553917) and 522 proteins in human TIG-3 cells (PMID: 36680776). This analysis revealed several shared replication-associated factors including RFC1-5, PCNA, MCM2-7) but also highlighted important differences between the proteomes at replication forks and at sites of UV damage repair with replication-associated factors that are not retrieved at sites of UV damage repair such as Cdc45, Claspin, MCM10, TONSL (Supplementary Fig.3c).

- Thank you for including this analysis in the revised manuscript. Please include the source data for the Venn Diagram in the supplemental excel file since it would be useful for the field to have the lists of proteins identified in each section of the Venn Diagrams depicted in Supplementary Figure 3b-c.

Beyond this, I was not sufficiently convinced by the follow-up studies whereby DNAJC9 was selected as the key factor in this process despite being close to the cut-off for statistical significance in the proteomic datasets. The most convincing aspect of this was that depletion of DNAJC9 causes a loss of new SNAP tagged H3.3/H3.1. In the UV irradiated experiments, the signal of DNAJC9 and MCM2 at irradiated sites was not convincing, no easily observable foci are formed. Furthermore, since this could also be explained by bleed through from the GFP channel into the red channel, and I was not able to find evidence that this has been correctly controlled for, this calls into doubt the validity of conclusions made based on this approach – thus at this stage it is unclear of the significance of many findings.

DNAJC9 was chosen with MCM2 because those were the only two histone chaperones in the IPOND-R capture that had not previously been involved in the UV damage response. Even if close to the cut-off for statistical significance, DNAJC9 recruitment was confirmed at UV sites by imaging approaches (Fig. 3e-f). We acknowledge that the observed enrichment of DNAJC9 at UV sites is rather mild and this is likely due to the fact that DNAJC9 is loosely bound to chromatin contrary to CAF-1 and HIRA chaperones (Supplementary Fig. 6n). MCM2 recruitment is more readily detectable at late time points post irradiation (Fig. 6a) and we verify that the observed histone chaperone signal enrichment at UV damage sites does not result from a bleed-through from the UV damage channel (new Supplementary Fig. 7a). To consolidate our findings, we examine more thoroughly the recruitment of DNAJC9 and MCM2 to sites of UV damage repair at different time points following damage infliction (Fig. 3e-f, Supplementary Fig. 4a, Fig. 6a-b) and we demonstrate that their recruitment to UV sites occurs outside of S phase (Supplementary Fig. 4b). DNAJC9 recruitment to UV damage sites 8h post irradiation was not very convincing and we thus decided to remove the data from the manuscript.

Functionally, our follow-up studies demonstrate that DNAJC9 affects histone H3 dynamics at repair sites, which we confirmed and further characterized through functional rescue experiments (Fig. 4d). These experiments establish that DNAJC9, through its histone binding domain, promotes the deposition of newly synthesized H3 variants at UV damage sites. In addition, we have run additional control experiments showing that the function of DNAJC9 in new histone deposition at repair sites does not require the passage of cells through S phase (Supplementary Fig. 7e).

- The data in figure 4d shows quite clearly that exogenous DNAJC9 expression does rescue new H3.3 levels at UVC DNA repair sites and this is not observed in the 4A histone binding mutant. This is an excellent addition to the manuscript.

Furthermore, the statistical handling of data is questionable. Whilst microscopy experiments have been performed with three biological replicates, paired t-tests appear to use all data points from these experiments rather than the three means of the biological replicates. This is a very confusing way to present the data and makes it difficult to assess how robust the conclusions made are. For example, are the changes in Figure 5 really strong enough to conclude that DNAJC9 has a role in old histone recovery/histone recycling?

Together, whilst I believe this is a promising story, it is not yet sufficiently developed for publication in Nature Comms.

We run the statistical analyses on all data points and not on the means of the biological replicates in conditions of local UV irradiation because in this case each nucleus receives a different UV dose, which depends on the pore distribution in the micropore filters used for local UVC irradiation (mostly 1 or 2 and up to 5 UV spots per nucleus, PMID: 39820273), so each nucleus should be considered independently. This is now stated in the method section (p. 35).

- On page 6 the authors state that “Damage induction is quite homogenous in a cell population (Supplementary Fig. 2c) so this does not introduce any bias in subsequent analyses.” This is at odds with the statistical handling of the data in Figure 5 and 6, where all data points across three biological replicates are used instead of the three biological replicate means during statistical testing. In addition, since the above critique would have been an easy point to rebut by simply recalculating P

values based on the three biological replicate means, does this mean that maintaining the current statistical representation is the only way to maintain the significance of this observation?

- Figure 4d should have indication of the UVC treatment in the figure (time post local UVC), similar to 4b.

Even if modest, the defects observed upon knockdown of DNAJC9 are comparable to the defects observed upon depletion of the HIRA and CAF-1 chaperones for new histone deposition (Fig. 4h-i) and comparable to the defects observed upon depletion of the late repair factor XPG for old histone recovery (Fig. 5b). Furthermore, the impaired recovery of old histones observed upon DNAJC9 depletion cannot be attributed to defective UV damage repair because DNAJC9-knocked down cells are fully proficient for the repair of UV lesions (Supplementary Fig. 5). This is now explained on p. 14 of our revised manuscript.

- In addition, why does siLuc in Figure 5b promote over-recovery of the TMR mean intensity at UVC sites/nucleus (the mean is >1)? In contrast with siDNAJC9 and siXPG the TMR mean intensity at UVC sites/nucleus is approx. =1, wouldn't this be consistent with full signal recovery? Therefore, wouldn't this data argue that DNAJC9 and XPG have no role in old histone recovery after UV damage. I therefore question the interpretation of the data here, and question whether this analysis is actually worthy of inclusion in the publication. I would consider the analysis in Supplementary Figure 7f as more pertinent to the story, this panel demonstrates the role of MCM2 in new H3.3 deposition at UVC sites, a novel discovery that should be given more prominence.

To further strengthen the role of DNAJC9 and MCM2 in old histone recovery, we ruled out the hypothesis that MCM2 and DNAJC9 contribution to old H3.3 recovery may be an indirect consequence of these chaperones stimulating new H3.3 deposition. Indeed, depletion of the H3.3 chaperone HIRA, known to deposit new H3.3 histones at sites of UV damage repair (PMID: 24074863), does not impair old H3.3 recovery (Supplementary Fig. 7g).

- This is an independent experiment, as such an experiment that included siRNA depletion of DNAJC9 and MCM2 would be more convincing. E.g. in the same experiment comparing.

- 1) siControl
- 2) siDNAJC9
- 3) siMCM2
- 4) siHIRA
- 5) siDNAJC9+siMCM2
- 6) siDNAJC9+siHIRA
- 7) siMCM2+siHIRA

- One final point is that the conclusion that siDNAJC9 affects the proportion of cells in S phase (Supplementary figure 6h) is based on the proportion of cells that are positive for the histone chaperone Caf1. Can this result be repeated with another more classical readout? E.g. FAC analysis, or using microscopy read-outs e.g. BrdU or EdU?

Version 2:

Reviewer comments:

Reviewer #5

(Remarks to the Author)

I am satisfied that the authors have now addressed my concerns.

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Point-by-point response to reviewer comments

We thank all five referees for insightful and constructive suggestions that proved very useful to improve our manuscript. As main additions to the manuscript:

- we provide additional comparisons of IPOND-R data to IPOND and aniFOUND
- we consolidate the recruitment of DNAJC9 and MCM2 to UV-damaged chromatin and assess their function in the UV damage response through functional rescue experiments
- we demonstrate that the effects observed are replication-independent by using alternative experimental approaches including G1-arrest with Palbociclib.

Reviewer #1:

The current manuscript presents a development of the IPOND technique aimed at capturing the proteome at UVC damage repair sites (IPOND-Repair). This is an interesting new technique that expands the arsenal of methods to study DNA repair in the context of chromatin, and could be further applied to explore the proteome at sites of diverse DNA lesions that require DNA synthesis for their resolution. Using IPOND-R, the authors identify ~250 proteins enriched at UVC damage sites (one-hour post-irradiation). These include several histone chaperones (DNAJC9, MCM2, CAF1, FACT...), nucleosome remodelling enzymes and histone modifiers. The work then focuses on the function of DNAJC9 and MCM2 at UVC damage sites. Chromatin is dynamically remodelled at UVC damage sites, including the transient displacement and recovery of 'old' histones, and the deposition of 'new' histones. A full picture of which histone chaperones are involved in this remodelling of chromatin is missing, and this work represents an important advance. Here, the authors show that DNAJC9 is required for incorporation of new histones H3 (H3.1 and H3.3 variants) at UVC damage sites (as well as their genome-wide incorporation); and that DNAJC9 and MCM2 similarly function in the recovery of old histone H3.3 levels at UVC repair sites. Together, the work implicates DNAJC9 and MCM2 in the maintenance of chromatin structure at UVC damage sites, without affecting the actual repair of UV lesions.

The main point that would greatly strengthen the manuscript is a deeper mechanistic understanding of how DNAJC9 and MCM2 function at sites of DNA damage. First, the establishment of a functional rescue system will be key to unequivocally assign the function of DNAJC9 and MCM2 during chromatin remodelling at UVC damage sites. We had established a functional rescue system for DNAJC9 (Supplementary Fig. 6d now with 3 experiments instead of 2) and we now provide a rescue experiment also for MCM2 showing that re-expression of wild-type MCM2 restores at least partially new H3.3 deposition at UV sites (Suppl Fig. 7f).

Second, as also pointed out by the authors, based on the current literature a number of mechanisms can be invoked to explain how DNAJC9 and MCM2 accumulate at sites of DNA damage to promote histone incorporation. These could be direct binding to histone H3-H4 (histones evicted from chromatin could become 'visible' to these chaperones?); interaction of MCM2 with other CMG components; regulation of 'damaged' chromatin binding by the J domain of DNAJC9. Importantly, point mutants that disrupt these interactions have all been described. The contribution of these mechanisms could be tested in a rescue system, and would transform our understanding of how DNAJC9 and MCM2 function at DNA repair sites.

Through functional rescue experiments, we have tested the impact of DNAJC9 histone binding mutant on the recruitment of this chaperone to sites of UV damage repair and on the resulting repair-coupled histone deposition. We initially planned to test both a histone binding mutant of DNAJC9 and a mutant in the J domain, which abolishes the interaction with Hsp70 family members (PMID: 33857403) but we could not achieve stable expression of the J domain mutant. We observed that expression of wild type DNAJC9 rescued, at least in part, new H3.3 deposition at UV damage sites in DNAJC9-knocked down cells, which was not observed upon expression of a histone binding mutant form of DNAJC9 although both wild type and mutant DNAJC9 were similarly recruited to UV damage sites (new Fig. 4d). These experiments establish that

DNAJC9, through its histone binding domain, promotes the deposition of newly synthesized histones at UV damage sites.

As for MCM2, the H3-H4 histone binding mutant (Y81A, Y90A) is not a perfect separation of function mutant because all MCM2 histone binding mutants also affect the assembly of the CMG replicative helicase complex to some extent (PMID: 26167883). Moreover, stable expression of the MCM2 histone binding mutant was highly cytotoxic, preventing functional rescue experiments with this construct. Note that while the whole MCM hexamer is captured at sites of UV damage repair, other CMG components like Cdc45 and GINS are not (Supplementary Fig. 3c), which indicates that MCM2 likely operates independently of the CMG complex at repair sites (now discussed on p. 17).

To further characterize MCM2 and DNAJC9 function in histone dynamics at repair sites, we ruled out the hypothesis that MCM2 and DNAJC9 contribution to old H3.3 recovery may be an indirect consequence of these chaperones stimulating new H3.3 deposition. Indeed, depletion of the H3.3 chaperone HIRA, known to deposit new H3.3 histones at sites of UV damage repair (PMID: 24074863), did not impair old H3.3 recovery (Supplementary Fig. 7g).

Other points:

Line 116: Figure S1E only shows XPB recruitment, not 'UV damage dependent factors', so the text should be amended accordingly.

We have rephrased this part (p. 6)

Fig. 2D is not very informative, particularly the 'reference number' for the gene ontology terms. Can this panel be reshaped?

We have removed the GO term reference numbers from the representation of the gene ontology analysis in Fig. 2d and included instead additional broad terms for the most enriched GO terms.

How do the authors explain the difference in proteins retrieved by aniFOUND as compared to IPOND-R (Fig. 3A)? adding a short explanation in the text would be helpful for readers to compare the two techniques. And how is the overlap between IPOND-R and IPOND (untreated conditions)?

The modest overlap with the aniFOUND dataset can be explained by the different cell types (U2OS osteosarcoma cells for IPOND-R vs. 1BR3 skin fibroblasts for aniFOUND) and most likely by the different time points post UV (1 h for IPOND-R vs. 4 h for aniFOUND) as early repair responses are not captured 4 h post irradiation (now explained on p. 8).

We have also compared the IPOND-R mass spectrometry dataset (1 h-specific hits in U2OS cells) to the proteins identified on nascent replicated strands in undamaged conditions by IPOND combined with mass spectrometry (593 proteins across 4 human cell lines HEK293T, HCT-116, HeLa and RPE-1 (PMID: 31553917) and 522 proteins in human TIG-3 cells (PMID: 36680776). This analysis revealed several shared replication-associated factors including RFC1-5, PCNA, MCM2-7 but also highlighted important differences between the proteomes at replication forks and at sites of UV damage repair with replication-associated factors that are not retrieved at sites of UV damage repair such as Cdc45, Claspin, MCM10, TONSL (Supplementary Fig.3c).

In Figure 4, the pattern of H3.1 incorporation into chromatin is used to define whether

cells are in S phase or not. However, given that loss of DNAJC9 decreases new H3.1-SNAP incorporation and the percentage of S-phase cells (Fig. S5G-I), how reliable is the observed H3.1 pattern? Can the authors test whether DNAJC9-depleted cells still consistently show the expected 'S-phase' (early, mid, late) patterns for H3.1-SNAP, p150 and/or EdU? This would validate the use of H3.1-SNAP patterns as a marker of S-phase in Fig. 4.

We can easily discriminate between cells in and outside S phase based on new H3.1 nuclear levels even in DNAJC9-knocked down cells because the levels of new H3.1 are always significantly higher in S phase cells (Supplementary Fig. 6a, S phase cells were identified based on CAF-1 p60, which is unchanged upon DNAJC9 knockdown).

Fig. 4H: Given that DNACJ9 and HIRA depletion leads to lower H3.3-SNAP incorporation, the H3.3-SNAP images seem not very representative. Has the contrast been changed differently between samples to highlight the decrease in H3.3-SNAP accumulation specifically at UVC sites? if so, this should be stated in the legend. The image settings are kept the same between samples. We now show more representative pictures that better reflect the results of the quantification presented on the graph (Fig. 4i).

Reviewer #2:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3:

The paper by Plessier et al, describes a new proteomic method to isolate factors accumulating on newly synthesized DNA after UV DNA damage, named IPOND-R. After validation of the method, the authors focus on analyzing the effect of depletion of DNAJC9 on the dynamics of new and old histones at DNA damage sites. The authors find that DNAJC9 controls the dynamics of both histone populations, but does not affect DNA damage repair per se (i.e. recruitment of repair proteins, or survival after DNA damage). The authors conclude by suggesting an interplay between MCM2 and DNAJC9 to control chromatin dynamics at UV damage sites. The paper contains high quality analyses of the effect of DNAJC9 depletion, and also provides a well validated new approach to study the proteome of DNA damage repair sites. In general, the findings are interesting and may attract attention from several research communities involved in DNA repair and chromatin biology. However, there are few concerns that need to be addressed before publication.

The most critical issue is that it is not clear that the effects described are direct and related to DNA damage repair, rather than indirect effects from the cells going through S-phase without critical histone chaperones. DNAJC9, MCM2 as well as CAF-1 and HIRA are critical histone chaperones and they will alter chromatin dynamics independently of DNA damage, as previously shown. Because the authors do not see an effect of DNAJC9 on DNA repair or survival after damage, it could be that the authors are simply measuring changes in chromatin dynamics that are not dependent on DNA damage, but related to global chromatin alterations. This is due in part to the experimental setup of the assay: the use of siRNA and the conditions used to inhibit DNA replication. Controls should be added to address this concern.

1) The authors run many important controls to validate the IPOND-R approach to minimize the contribution of DNA replication in the dataset. However, treatment of cells with HU and Ara-C to inhibit replicative synthesis also stresses these cells significantly. I wonder if the authors could use synchronization of the cells in G1/S by Palbociclib (which is known to not induce stress) to conclusively demonstrate the power of the method. Running IPOND-R and the follow up (key) experiments in Palbociclib arrested cells will provide important confirmation that DNA replication and S-phase mechanisms are not at play in the chromatin dynamics presented here.

The combination of HU and ara-C indeed triggers genotoxic stress. To address this valid concern, we used instead the cdk4/6 inhibitor Palbociclib to arrest cells in G1. This treatment was applied to RPE-1 cells because U2OS cells are resistant due to high cdk4 expression. We confirmed by EdU staining the complete depletion of replicating cells upon Palbociclib treatment while repair synthesis remained unaffected. However, we noticed that Palbociclib treatment hindered the UV-induced stress response as evidenced by a lack of increase of γ H2A.X signal between non-irradiated and UV-irradiated cells, preventing us from running successful IPOND-R experiments in Palbociclib-treated cells (no enrichment of material in the +UV capture, see Figure for Reviewer below). Nevertheless, we could monitor repair-coupled histone dynamics, such as new H3.3 deposition, in cells arrested in G1 by Palbociclib treatment. Similar to what observed in cycling cells, DNAJC9 and MCM2 knock-down in G1-arrested cells impaired new H3.3 deposition at sites of UV damage repair (Supplementary Fig. 7e), arguing that the function of these chaperones in histone deposition coupled to repair does not require passage through S phase. Furthermore, DNAJC9 and MCM2

recruitment to UV damaged chromatin was observed outside of S phase (Supplementary Fig. 4b), indicating that they are replication-independent.

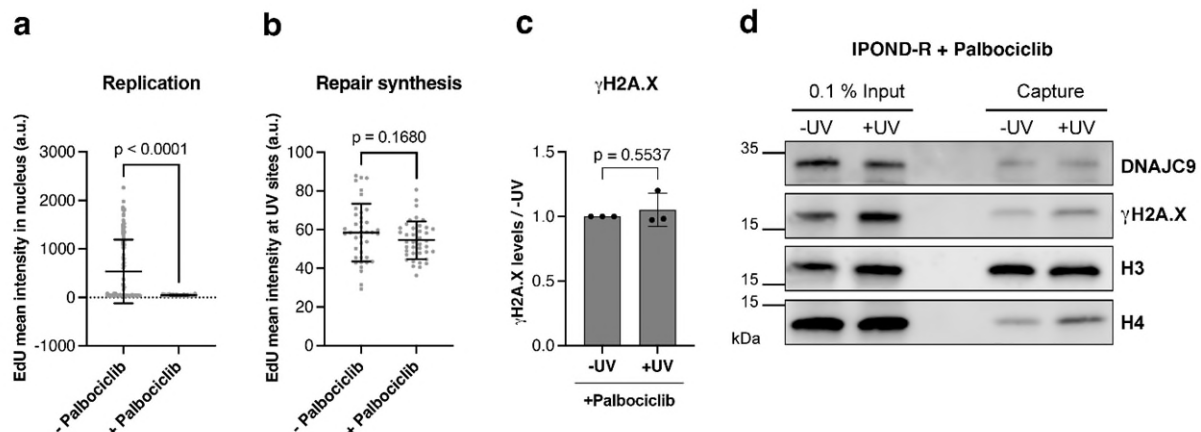


Figure for Reviewer: Set up of IPOND-R in cells arrested in G1 by Palbociclib treatment

(a) Inhibition of replication-coupled DNA synthesis measured by EdU incorporation (pulse of 10 min) in RPE-1 cells treated for 24 h with 500 nM Palbociclib.

(b) Effect of Palbociclib pre-treatment on repair-coupled DNA synthesis measured by EdU incorporation (pulse of 1 h) at sites of local UV irradiation (marked by CPD immunostaining) in RPE-1 cells. S phase cells present in the non-treated population were identified by pan-nuclear EdU staining and excluded from the analysis.

(c) γ H2A.X levels analyzed by western blot in total extracts from RPE-1 cells treated for 24 h with 500 nM Palbociclib and exposed or not to 10 J/m² global UV irradiation. Cells were harvested 1 h post irradiation.

(d) Western blot analysis of proteins captured by IPOND-R 1 h post UV irradiation in RPE-1 cells arrested in G1 by 24 h treatment with 500 nM Palbociclib. -UV is used as negative control.

Dot plots: mean $\pm$ SD. Statistical significance is calculated with a two-sided Student's t-test with Welch's correction.

2) As the authors deplete their proteins with siRNA, the cells are cycling in the absence of the target proteins for at least one full cell cycle. As the authors are targeting critical histone chaperones (DNAJC9, MCM2, CAF-1 and HIRA), it is expected that chromatin is already affected at the time of DNA damage induction. The authors should find ways to circumvent this experimental limitation (i.e. use rapid degron-based degradation or test their key findings in G1 arrested cells).

As explained above, we validated our findings in G1-arrested RPE-1 cells by palbociclib treatment and thus confirmed the contribution of DNAJC9 and MCM2 to repair-coupled histone deposition, independently of cell passage through S phase (Supplementary Fig. 7e).

3) In line 228-229 it is stated that the quantification of Figure 4B focuses on non S-phase cells. It is not clear to this reviewer how one can reliably distinguish S and non S-phase cells from new H3.1 distribution (I can only imagine a difference between early and late S-phase). I expect a difference could be in the total amount of new H3.1 (should be significantly higher in S-phase cells), but not clear how the distribution can alone explain S vs non-S phase cells. At any rate, the authors should include a quantification for S-phase cells to test if there is a difference. Could the authors use EdU to distinguish various populations otherwise? Or synchronize with Palbociclib as suggested above?

We can easily discriminate between cells in and outside S phase based on new H3.1 nuclear levels even in DNAJC9-knocked down cells because the levels of new H3.1 are always significantly higher in S phase cells (Supplementary Fig. 6a, S phase cells were identified based on CAF-1 p60, which is unchanged upon DNAJC9 knockdown).

4) The involvement of MCM2 is also unusual, without evoking the passage of DNA replication forks. The authors do not really mention this, but this needs to be addressed/discussed. How can they envision MCM2 working at these sites, or together with DNAJC9, outside of DNA replication? Hypotheses need to be tested to clarify their data, which is very preliminary in the current form.

MCM2 is enriched in the IPOND-R dataset together with the other MCM subunits (MCM3 to 7) showing that the whole MCM hexamer is present at sites of UV damage repair like at replication forks. However, the observed recruitment of MCM2 to sites of UV damage repair does not require the passage of replication forks because it is detected outside of S phase (new Supplementary Fig. 4b), highlighting a function of MCM2 that is disconnected from DNA replication. In line with this, we do not retrieve other components of the CMG replicative helicase complex at repair sites like Cdc45 and GINS subunits (Supplementary Fig. 3c, Supplementary Table 1), which indicates that MCM2 likely operates independently of the replicative helicase complex at repair sites. Notably, an abundant pool of MCM proteins is not engaged with active replisomes in human cells (PMID: 36241664) and those could fulfill replication-independent roles (now discussed on p.17-18).

5) In Figure 1D, the authors should probe for DNA replication proteins to show that they are not pulled down (e.g. Cdc45, GINS, or fork protection complex components). The comparison between IPOND-R and IPOND data sets (see point 6 below) reveals important differences between the proteomes at replication forks and at sites of UV damage repair. Some replication-associated proteins are not captured at repair sites including Claspin, Cdc45, MCM10, TONSL (Supplementary Fig. 3c). We confirmed by western blot that the replication-associated protein Cdc45 was not enriched in the +UV capture (Supplementary Fig. 3d).

6) The authors should also show the overlap between IPOND-R and previously reported IPOND hits (in Figure 2), to show how many of the fork proteins are present in the data.

We have compared the IPOND-R mass spectrometry dataset (1 h-specific hits in U2OS cells) to the proteins identified on nascent replicated strands in undamaged conditions by IPOND-mass spectrometry (593 proteins across 4 human cell lines HEK293T, HCT-116, HeLa and RPE-1 (PMID: 31553917) and 522 proteins in human TIG-3 cells (PMID: 36680776). This analysis revealed several shared replication-associated factors including RFC1-5, PCNA, MCM2-7 but also highlighted important differences between the proteomes at replication forks and at sites of UV damage repair with replication-associated factors that are not retrieved at sites of UV damage repair such as Claspin, Cdc45, MCM10, TONSL (Supplementary Fig.3c)

There is an instance of “data not shown” in the discussion (lines 339-340). These data should be shown, or the statement needs to be removed.

We now show that DNAJC9 is loosely bound to UV-damaged chromatin, as it is mostly solubilized by detergents, in contrast to CAF-1 and HIRA (Supplementary Fig. 6n).

Reviewer #4:

The study investigates chromatin dynamics during UV damage repair, focusing on identifying proteins that regulate histone deposition at damaged sites. The authors modified the IPOND proteomic method, naming it IPOND-R, to analyze protein dynamics specific to repair sites. Through this approach, they identified DNAJC9 and MCM2, which had not previously been linked to UV-induced damage. DNAJC9 is suggested to facilitate the deposition of new histones and recovery of parental histones, while MCM2 is proposed to collaborate in these processes to maintain chromatin integrity. This finding adds to the understanding of the dynamic regulation of chromatin following DNA damage. However, several issues undermine the study's conclusions. Many results lack appropriate controls, and the experimental descriptions are insufficiently detailed, making the findings difficult to interpret and raising concerns about their reliability. Furthermore, the logical connections between key results are unclear. These shortcomings lead to ambiguous conclusions, and some interpretations appear to extend beyond what the data can support.

Major:

1. Based on the proposed model of chromatin repair (Figure 6F), it appears that DNA breaks are repaired first, followed by histone deposition to restore chromatin structure. However, the authors did not assess when DNA repair is complete. Notably, the persistence of γ H2AX after 24 hours suggests that repair may not be fully resolved, and DNAJC9 is no longer present at the damage sites at this time point. Furthermore, while DNAJC9 is transiently present at UVC damage sites, the authors report no evidence of its involvement in DNA repair. These findings raise important questions about the proposed model and the role of DNAJC9 at DNA damage sites. To clarify these points and strengthen the model, the authors should provide additional details, including:

- Evidence indicating when DNA repair is complete.
- Whether the loss of DNAJC9 affects transcription recovery near break sites following UVC damage.

Addressing these points will help establish a clearer connection between DNA repair, histone deposition, and the function of DNAJC9 in the context of DNA damage.

Some histone deposition arises before DNA repair completion as shown for new H3.3 deposition, which is coupled to early repair steps, namely UV damage detection (PMID: 24074863). To better illustrate this point, we now indicate on the model in Fig. 6f “New histone deposition coupled to DNA repair”. Therefore, the presence of a histone chaperone on UV-damaged chromatin before repair completion does not preclude an involvement of this chaperone in chromatin restoration coupled to DNA repair.

Evidence for the completion of UV damage repair around 24 h post UV is two-fold: (1) The kinetics of UV lesion removal (Supplementary Fig. 1f-g) shows that 6,4-photoproducts are repaired fast (within 2 h) and the CPDs are removed in 24-30 h. (2) UV damage repair factors are no longer captured by IPOND-R 24 h post UV indicating that most repair is complete by that time. The persistence of some γ H2A.X signal 24 h post UV (Fig. 3d) is not a direct readout of the presence of DNA damage. It shows that DNA damage signaling is not yet turned off, which occurs after repair completion.

To further characterize the function of DNAJC9 in UV-damaged chromatin, we investigated the contribution of this histone chaperone to transcription recovery following UVC damage by labeling nascent transcripts with ethynyl-uridine. Knocking

down DNAJC9 did not impair transcription recovery at UV damage sites in contrast to knocking down the transcription-coupled repair factor CSB (Supplementary Fig. 5f). These results are consistent with a previous multiomic analysis of the UV damage response (PMID: 27184836) where DNAJC9 did not stand out as a factor regulating nascent transcription post UV, and rule out a function of DNAJC9 in transcription-coupled repair.

2. The authors treated cells with 10 mM HU and 1 mM AraC to inhibit DNA replication, raising several concerns:

- While AraC can inhibit DNA replication, it is also incorporated into repair sites, potentially causing repair defects. This issue is not addressed in the manuscript, undermining the validity of the experimental approach.
- The use of 1 mM AraC is an extremely high concentration for dividing cells, especially since concentrations as low as 40 μ M can be toxic to some post-mitotic cells. The observation that cells accumulate in the G1 phase even 24 hours post-treatment with DNA synthesis inhibitors suggests that the inhibitors themselves may have induced significant cellular stress.

Given these concerns, we recommend replacing HU+AraC with other inhibitors, such as G1 arrest inhibitors (eg. Palbociclib), which would allow the cells to arrest in G1 phase and prevent EdU incorporation during DNA replication. This adjustment would enhance the reliability of the approach and reduce potential confounding effects.

Although we verified that the combination HU+Ara-C did not cause UV damage repair defects (Supplementary Fig. 1e-g), we acknowledge that such treatment can trigger genotoxic stress (Supplementary Fig. 1a-c). To address this valid concern, also raised by Reviewer #3, we used the cdk4/6 inhibitor Palbociclib to arrest cells in G1. This treatment was applied to RPE-1 cells because U2OS cells are resistant due to high cdk4 expression. We confirmed by EdU staining the complete depletion of replicating cells upon Palbociclib treatment while repair synthesis remained unaffected. However, we noticed that Palbociclib treatment hindered the UV-induced stress response, as evidenced a lack of increase of γ H2A.X signal between non-irradiated and UV-irradiated cells, preventing us from running successful IPOND-R experiments in Palbociclib-treated cells (no enrichment of material in the +UV capture, see Figure for Reviewer below). Nevertheless, we could monitor repair-coupled histone dynamics, such as new H3.3 deposition, in cells arrested in G1 by Palbociclib treatment. Similar to what observed in cycling cells, DNAJC9 and MCM2 knock-down in G1-arrested cells impaired new H3.3 deposition at sites of UV damage repair (Supplementary Fig. 7e), arguing that the function of these chaperones in histone deposition coupled to repair does not require passage through S phase.

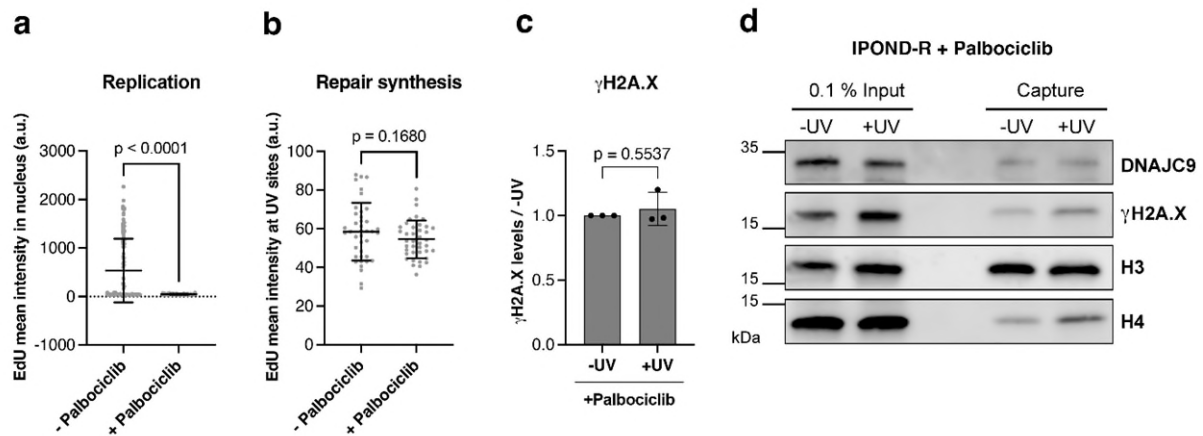


Figure for Reviewer: Set up of IPOND-R in cells arrested in G1 by Palbociclib treatment

(a) Inhibition of replication-coupled DNA synthesis measured by EdU incorporation (pulse of 10 min) in RPE-1 cells treated for 24 h with 500 nM Palbociclib.

(b) Effect of Palbociclib pre-treatment on repair-coupled DNA synthesis measured by EdU incorporation (pulse of 1 h) at sites of local UV irradiation (marked by CPD immunostaining) in RPE-1 cells. S phase cells present in the non-treated population were identified by pan-nuclear EdU staining and excluded from the analysis.

(c) γ H2A.X levels analyzed by western blot in total extracts from RPE-1 cells treated for 24 h with 500 nM Palbociclib and exposed or not to 10 J/m² global UV irradiation. Cells were harvested 1 h post irradiation.

(d) Western blot analysis of proteins captured by IPOND-R 1 h post UV irradiation in RPE-1 cells arrested in G1 by 24 h treatment with 500 nM Palbociclib. -UV is used as negative control.

Dot plots: mean $\pm$ SD. Statistical significance is calculated with a two-sided Student's t-test with Welch's correction.

3. In Figure 5B, siXPG should prevent UV lesion removal, thereby halting DNA repair. However, the data still show a recovery of old H3.3, which is confusing. If DNA repair is blocked, it would be expected to also impact histone deposition. The authors should clarify how histone deposition occurs independently of DNA repair in this context and provide additional insights into DNAJC9's function.

In Fig. 5b, siXPG is indeed used as a positive control because XPG depletion prevents UV lesion removal thus impacting old histone recovery. XPG knock-down still allows some old histone recovery because the siRNA-mediated knock-down does not fully remove the protein thus repair is not 100% abrogated (as now stated on p. 13-14) but old histone recovery is significantly impaired in siXPG compared to the siLuc control condition ($p < 0.0001$).

Minor:

1. To identify proteins that transiently bind to chromatin during repair, the authors compared IPOND-R results at 1-hour and 24-hour post UV time points. However, this approach does not rule out the possibility that cells with the most severe damage at 1 hour post-UV may not survive until the 24-hour time point, thus create bias. To address these concerns, the authors should perform EdU pull-down sequencing to confirm that the EdU-incorporated sites at 24 hours are comparable in abundance and distribution to those at 1 hour post-UV treatment.

We show that the levels of incorporated EdU in cell nuclei are not lower at 24 h compared to 1 h post UV (Supplementary Fig. 2b), arguing against the death of UV-damaged, EdU-labeled cells between 1 h and 24 h post irradiation (now stated on p.6).

Furthermore, the amount of UV damage is quite homogeneous in a cell population as shown by CPD staining immediately after global UVC irradiation (new Supplementary Fig. 2c) so there is not a large proportion of cells with more severe damage and it does not introduce any bias in the analysis.

2. The analysis in Figures 4 and 5 presents data at 20 minutes, 2 hours, and 8 hours post-UVC exposure without providing any explanation for this shift, despite earlier data focusing on 1 hour post-UVC. This raises questions about whether DNAJC9 remains present at UV damage sites at these time points in the control. To address these issues, the authors should provide a clear rationale for the time-point selection.

Twenty minutes post UV was previously determined as the best time point to visualize HIRA accumulation at UV sites, 2 h is the best time point to visualize CAF-1 and new H3 deposition at repair sites (PMID: 24074863) and 8 h is suited to visualize old histone recovery (PMID: 27642047). In addition to the 1 h and 8 h time points (Fig. 3e-f and 6a), we now also show DNAJC9 and MCM2 enrichment at sites of UV damage repair 20 min and 2 h following local UVC irradiation (Supplementary Fig. 4a).

3. The IF results for new H3.3-SNAP in siDNAJC9#3 in Figures 4C and 4H display inconsistent deposition patterns (completely absent in one instance and still present in another), which raises concerns about data reliability. The authors should use figures displaying multiple cells instead of single cells to ensure greater consistency and reliability in the presentation of the data.

We now show more representative pictures in Fig. 4i that better reflect the results of the quantification presented on the graph. We believe that quantifying the signal in at least 150 cells scored in 3 independent experiments is the most reliable way of presenting the data.

4. There is no description of how the survival assay was performed. It is unclear whether the UVC dose of 10 J/m² causes 85% cell death within 24 hours, 48 hours, or over the course of a week, which creates confusion and raises questions about the experimental conditions.

The description of the survival assay was present in the method section under the caption "Colony forming assays", which we have now rephrased as "Cell survival assays" for clarity. In these assays, cell colonies are counted after 12 days of growth following UV irradiation. As previously stated, there is hardly any detectable cell death within 24 h post UV.

In light of these concerns, the paper's conclusions about DNAJC9's role in chromatin repair appear premature and not fully supported by the data presented. A substantial revision addressing these issues would be necessary to strengthen the study's findings and interpretations.

Reviewer #5:

Plessier et al presents an innovative proteomic approach to capture chromatin in the process of UVC irradiation repair based on the IPOND protocol (IPOND-R). This protocol relies on the complete inhibition of DNA replication such that EdU is only incorporated during UV-damage coupled DNA repair processes. As can be seen in Figure 1B, the inhibition of DNA replication with HU and Ara-C combined is highly effective. Furthermore, the post UVC repair EdU signal is similar in non S-phase cells when not treated with these inhibitors (Figure 1C). These are important controls since small amounts of DNA replication could significantly skew the interpretation of the resulting proteomic analyses from these experiments. Whilst the proteomic datasets have merit, there should be a more detailed comparison and benchmarking of them compared to classical IPOND experiments. For example, by comparing them to the datasets in Sirbu et al JBC VOL. 288, NO. 44, pp. 31458 –3146 (2013). This would help to highlight the differences to factors identified at the replication fork to those enriched at sites of UV damage repair.

As suggested, we have compared the IPOND-R mass spectrometry dataset (1 h-specific hits in U2OS cells) to the proteins identified on nascent replicated strands in undamaged conditions by IPOND-mass spectrometry (593 proteins across 4 human cell lines HEK293T, HCT-116, HeLa and RPE-1 (PMID: 31553917) and 522 proteins in human TIG-3 cells (PMID: 36680776). This analysis revealed several shared replication-associated factors including RFC1-5, PCNA, MCM2-7) but also highlighted important differences between the proteomes at replication forks and at sites of UV damage repair with replication-associated factors that are not retrieved at sites of UV damage repair such as Cdc45, Claspin, MCM10, TONSL (Supplementary Fig.3c).

Beyond this, I was not sufficiently convinced by the follow-up studies whereby DNAJC9 was selected as the key factor in this process despite being close to the cut-off for statistical significance in the proteomic datasets. The most convincing aspect of this was that depletion of DNAJC9 causes a loss of new SNAP tagged H3.3/H3.1. In the UV irradiated experiments, the signal of DNAJC9 and MCM2 at irradiated sites was not convincing, no easily observable foci are formed. Furthermore, since this could also be explained by bleed through from the GFP channel into the red channel, and I was not able to find evidence that this has been correctly controlled for, this calls into doubt the validity of conclusions made based on this approach – thus at this stage it is unclear of the significance of many findings.

DNAJC9 was chosen with MCM2 because those were the only two histone chaperones in the IPOND-R capture that had not previously been involved in the UV damage response. Even if close to the cut-off for statistical significance, DNAJC9 recruitment was confirmed at UV sites by imaging approaches (Fig. 3e-f). We acknowledge that the observed enrichment of DNAJC9 at UV sites is rather mild and this is likely due to the fact that DNAJC9 is loosely bound to chromatin contrary to CAF-1 and HIRA chaperones (Supplementary Fig. 6n). MCM2 recruitment is more readily detectable at late time points post irradiation (Fig. 6a) and we verify that the observed histone chaperone signal enrichment at UV damage sites does not result from a bleed-through from the UV damage channel (new Supplementary Fig. 7a). To consolidate our findings, we examine more thoroughly the recruitment of DNAJC9 and MCM2 to sites of UV damage repair at different time points following damage infliction (Fig. 3e-f, Supplementary Fig. 4a, Fig. 6a-b) and we demonstrate that their recruitment to UV sites occurs outside of S phase (Supplementary Fig. 4b). DNAJC9 recruitment to UV

damage sites 8h post irradiation was not very convincing and we thus decided to remove the data from the manuscript.

Functionally, our follow-up studies demonstrate that DNAJC9 affects histone H3 dynamics at repair sites, which we confirmed and further characterized through functional rescue experiments (Fig. 4d). These experiments establish that DNAJC9, through its histone binding domain, promotes the deposition of newly synthesized H3 variants at UV damage sites. In addition, we have run additional control experiments showing that the function of DNAJC9 in new histone deposition at repair sites does not require the passage of cells through S phase (Supplementary Fig. 7e).

Furthermore, the statistical handling of data is questionable. Whilst microscopy experiments have been performed with three biological replicates, paired t-tests appear to use all data points from these experiments rather than the three means of the biological replicates. This is a very confusing way to present the data and makes it difficult to assess how robust the conclusions made are. For example, are the changes in Figure 5 really strong enough to conclude that DNAJC9 has a role in old histone recovery/histone recycling?

Together, whilst I believe this is a promising story, it is not yet sufficiently developed for publication in Nature Comms.

We run the statistical analyses on all data points and not on the means of the biological replicates in conditions of local UV irradiation because in this case each nucleus receives a different UV dose, which depends on the pore distribution in the micropore filters used for local UVC irradiation (mostly 1 or 2 and up to 5 UV spots per nucleus, PMID: 39820273), so each nucleus should be considered independently. This is now stated in the method section (p. 35).

Even if modest, the defects observed upon knockdown of DNAJC9 are comparable to the defects observed upon depletion of the HIRA and CAF-1 chaperones for new histone deposition (Fig. 4h-i) and comparable to the defects observed upon depletion of the late repair factor XPG for old histone recovery (Fig. 5b). Furthermore, the impaired recovery of old histones observed upon DNAJC9 depletion cannot be attributed to defective UV damage repair because DNAJC9-knocked down cells are fully proficient for the repair of UV lesions (Supplementary Fig. 5). This is now explained on p. 14 of our revised manuscript.

To further strengthen the role of DNAJC9 and MCM2 in old histone recovery, we ruled out the hypothesis that MCM2 and DNAJC9 contribution to old H3.3 recovery may be an indirect consequence of these chaperones stimulating new H3.3 deposition. Indeed, depletion of the H3.3 chaperone HIRA, known to deposit new H3.3 histones at sites of UV damage repair (PMID: 24074863), does not impair old H3.3 recovery (Supplementary Fig. 7g).

Point-by-point response to reviewer comments

We thank all referees for a careful analysis of our manuscript and for their additional suggestions, which we fully address below.

Reviewer #1:

The authors have answered most of the questions we raised. A couple of minor points need to be clarified in the text.

Fig. 4D: in the H3.3-SNAP panel, what are the authors measuring? Is it the total levels of H3.3-SNAP expression? In siLuc or siDNAJC9 cells (and if it is in the latter, how does that fit with DNAJC9 leading to overall decreased histone incorporation?)

We are measuring the total levels of H3.3-SNAP in nuclei in the absence of DNAJC9 knockdown. This is now indicated in the corresponding figure legend (p. 45).

On page 13, the authors state that 'DNAJC9 likely provides new H3-H4 histones to CAF-1 and HIRA chaperones because DNAJC9 is loosely bound to UV-damaged chromatin, as it is mostly solubilized by detergents'. While I agree that DNAJC9 likely acts upstream of HIRA and CAF1, I think that this cannot be inferred by a detergent extraction method. There is literature suggesting this, and I think that this sentence should be rephrased accordingly.

We have rephrased this sentence not to infer DNAJC9 upstream function in the histone supply chain solely based on detergent extraction (p.13).

Reviewer #2:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3:

The authors have done a good job at addressing my concerns, it's unfortunate that they could not perform IPOND-R after treatment with Palbociclib due to technical reasons. Nonetheless, the manuscript has significantly improved and now provides important contributions to the field, that should be published.

Reviewer #4:

The authors have adequately addressed most comments from the reviewers. I'm still somewhat concerned about whether the histone chaperones at sites of DNA repair at UV lesions is related to stalled replication forks. However, the authors tried to address this concern and the results are consistent with their hypothesis. Thus, I do recommend publication.

Reviewer #5:

- Please see comments below in red. Some are major concerns which are easily addressed, yet authors have not attempted to address them. Specifically, my main concerns are surrounding the significance of the data presented in Figure 5. I have detailed steps to address my concerns below, and these should be addressed prior to acceptance for publication.

- In addition to the steps below, I noted Sandra Piquet is missing from the author list in the manuscript file.

Sandra Piquet is not listed as an author because she did not perform experiments in this study. She is leading the microscopy facility and is therefore listed in the acknowledgement section.

As suggested, we have compared the IPOND-R mass spectrometry dataset (1 h-specific hits in U2OS cells) to the proteins identified on nascent replicated strands in undamaged conditions by IPOND-mass spectrometry (593 proteins across 4 human cell lines HEK293T, HCT-116, HeLa and RPE-1 (PMID: 31553917) and 522 proteins in human TIG-3 cells (PMID: 36680776). This analysis revealed several shared replication-associated factors including RFC1-5, PCNA, MCM2-7) but also highlighted important differences between the proteomes at replication forks and at sites of UV damage repair with replication-associated factors that are not retrieved at sites of UV damage repair such as Cdc45, Claspin, MCM10, TONSL (Supplementary Fig.3c).

- Thank you for including this analysis in the revised manuscript. Please include the source data for the Venn Diagram in the supplemental excel file since it would be useful for the field to have the lists of proteins identified in each section of the Venn Diagrams depicted in Supplementary Figure 3b-c.

We have included this data in Supplementary Table 2 (p.8).

- The data in figure 4d shows quite clearly that exogenous DNAJC9 expression does rescue new H3.3 levels at UVC DNA repair sites and this is not observed in the 4A histone binding mutant. This is an excellent addition to the manuscript.

We thank the reviewer for underlining this.

- On page 6 the authors state that “Damage induction is quite homogenous in a cell population (Supplementary Fig. 2c) so this does not introduce any bias in subsequent analyses.” This is at odds with the statistical handling of the data in Figure 5 and 6, where all data points across three biological replicates are used instead of the three biological replicate means during statistical testing. In addition, since the above critique would have been an easy point to rebut by simply recalculating P values based on the three biological replicate means, does this mean that maintaining the current statistical representation is the only way to maintain the significance of this observation?

A distinction needs to be made between global UV irradiation, which leads to homogeneous damage induction in the cell population (Supplementary Fig. 2c), and local UV irradiation (performed in Figures 5 and 6), where each nucleus receives a different UV dose, which depends on the pore distribution in the micropore filters. The statistical handling of the data is thus different between global and local UV irradiation

experiments. We now more clearly explain in the method section how we run the statistical analyses of the data in each case (p.35).

- Figure 4d should have indication of the UVC treatment in the figure (time post local UVC), similar to 4b.

This has been added (Fig. 4d).

- In addition, why does siLuc in Figure 5b promote over-recovery of the TMR mean intensity at UVC sites/nucleus (the mean is >1)? In contrast with siDNAJC9 and siXPG the TMR mean intensity at UVC sites/nucleus is approx. =1, wouldn't this be consistent with full signal recovery? Therefore, wouldn't this data argue that DNAJC9 and XPG have no role in old histone recovery after UV damage. I therefore question the interpretation of the data here, and question whether this analysis is actually worthy of inclusion in the publication. I would consider the analysis in Supplementary Figure 7f as more pertinent to the story, this panel demonstrates the role of MCM2 in new H3.3 deposition at UVC sites, a novel discovery that should be given more prominence.

Old histone recovery ideally is measured through live cell imaging post UVC laser damage by comparing old histone levels in the UV-damaged region at a late time point post irradiation to the condition before UV irradiation (PMID: 27642047). In this context, full recovery would give a value of 1 but this live imaging method is not feasible with multiple siRNAs. To measure old histone recovery in fixed cells instead, we used as a proxy the ratio of old histones at UV damage sites relative to the nucleus at an early (10 min) and a late time point (8 h) post UV irradiation. The ratio of old histones at UV damage sites relative to the nucleus 8 h post UV in siLuc cells sets the level of recovery of old histones in control condition (even if slightly above 1). Comparatively, old histone recovery is diminished in siXPG and siDNAJC9 conditions. This is now better explained in our revised manuscript (p.13).

We have also emphasized the results shown in Supplementary Fig. 7f in the main text (p.16).

To further strengthen the role of DNAJC9 and MCM2 in old histone recovery, we ruled out the hypothesis that MCM2 and DNAJC9 contribution to old H3.3 recovery may be an indirect consequence of these chaperones stimulating new H3.3 deposition. Indeed, depletion of the H3.3 chaperone HIRA, known to deposit new H3.3 histones at sites of UV damage repair (PMID: 24074863), does not impair old H3.3 recovery (Supplementary Fig. 7g).

- This is an independent experiment, as such an experiment that included siRNA depletion of DNAJC9 and MCM2 would be more convincing. E.g. in the same experiment comparing.

- 1) siControl
- 2) siDNAJC9
- 3) siMCM2
- 4) siHIRA
- 5) siDNAJC9+siMCM2
- 6) siDNAJC9+siHIRA
- 7) siMCM2+siHIRA

As shown below (Fig. R1), we had performed siDNAJC9 as a control in the same experiment as siHIRA and observed that while siDNAJC9 reduced old H3.3 recovery,

consistent with previous experiments, siHIRA did not diminish old H3.3 recovery. We decided to focus on siHIRA and did not include the siDNAJC9 data in Suppl. Fig. 7g because it already appeared in other figure panels (Fig. 6c-d).

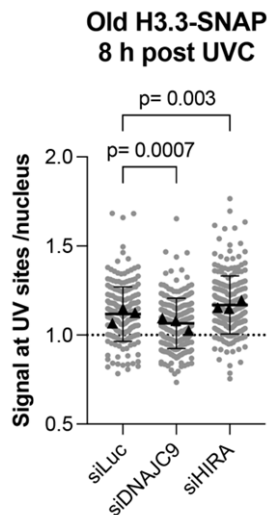


Figure R1: Quantification of old H3.3-SNAP histone enrichment at sites of UVC irradiation (marked by CPD) relative to the nucleus in U2OS cells stably expressing SNAP-tagged H3.3 and treated with the indicated siRNAs (siLuc, negative control). Mean $\pm$ SD from cells scored in 3 independent experiments (the mean of each experiment is represented by a grey triangle). Statistical significance is calculated by one-way ANOVA with multiple comparison.

- One final point is that the conclusion that siDNAJC9 affects the proportion of cells in S phase (Supplementary figure 6h) is based on the proportion of cells that are positive for the histone chaperone Caf1. Can this result be repeated with another more classical readout? E.g. FAC analysis, or using microscopy read-outs e.g. BrdU or EdU?

We scored S-phase cells based on CAF-1 staining in Suppl. Fig. 6h because we verified that siDNAJC9 did not affect CAF-1 levels (western blot in Suppl. Fig. 6b) and we obtained comparable results by scoring S phase cells based on EdU staining (Fig. R2). We now state this in the corresponding figure legend.

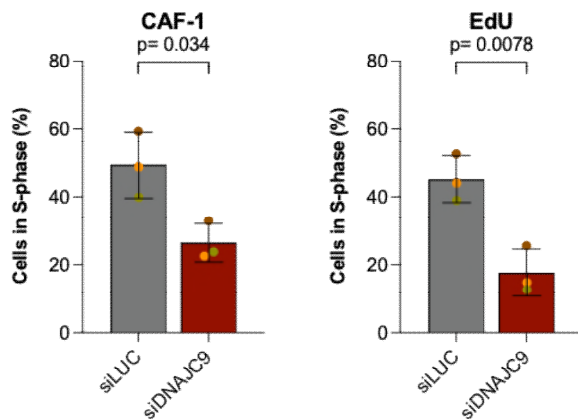


Figure R2: Percentage of cells in S-phase determined by CAF-1 p60 immunostaining of replication foci (left) or by EdU nuclear staining (right) in U2OS cells treated with the indicated siRNAs (siLuc, control). Mean $\pm$ SD from cells scored in 3 independent experiments. Statistical significance is calculated with a two-sided Student's t-test with Welch's correction.

Please see comments below in red. Some are major concerns which are easily addressed, yet authors have not attempted to address them. Specifically, my main concerns are surrounding the significance of the data presented in Figure 5. I have detailed steps to address my concerns below, and these should be addressed prior to acceptance for publication.

In addition to the steps below, I noted Sandra Piquet is missing from the author list in the manuscript file.

Point-by-point response to reviewer comments

We thank all five referees for insightful and constructive suggestions that proved very useful to improve our manuscript. As main additions to the manuscript:

- we provide additional comparisons of IPOND-R data to IPOND and aniFOUND
Please include source data for the Venn Diagram in the supplemental excel file. It would be useful for the field to have the lists of proteins identified in each section of the Venn Diagrams depicted in Supplementary Figure 3b-c.
- we consolidate the recruitment of DNAJC9 and MCM2 to UV-damaged chromatin and assess their function in the UV damage response through functional rescue experiments
- we demonstrate that the effects observed are replication-independent by using alternative experimental approaches including G1-arrest with Palbociclib.

Reviewer #1:

The current manuscript presents a development of the IPOND technique aimed at capturing the proteome at UVC damage repair sites (IPOND-Repair). This is an interesting new technique that expands the arsenal of methods to study DNA repair in the context of chromatin, and could be further applied to explore the proteome at sites of diverse DNA lesions that require DNA synthesis for their resolution. Using IPOND-R, the authors identify ~250 proteins enriched at UVC damage sites (one-hour post-irradiation). These include several histone chaperones (DNAJC9, MCM2, CAF1, FACT...), nucleosome remodelling enzymes and histone modifiers. The work then focuses on the function of DNAJC9 and MCM2 at UVC damage sites. Chromatin is dynamically remodelled at UVC damage sites, including the transient displacement and recovery of 'old' histones, and the deposition of 'new' histones. A full picture of which histone chaperones are involved in this remodelling of chromatin is missing, and this work represents an important advance. Here, the authors show that DNAJC9 is required for incorporation of new histones H3 (H3.1 and H3.3 variants) at UVC damage sites (as well as their genome-wide incorporation); and that DNAJC9 and MCM2 similarly function in the recovery of old histone H3.3 levels at UVC repair sites. Together, the work implicates DNAJC9 and MCM2 in the maintenance of chromatin structure at UVC damage sites, without affecting the actual repair of UV lesions.

The main point that would greatly strengthen the manuscript is a deeper mechanistic understanding of how DNAJC9 and MCM2 function at sites of DNA damage. First, the establishment of a functional rescue system will be key to unequivocally assign the function of DNAJC9 and MCM2 during chromatin remodelling at UVC damage sites.

We had established a functional rescue system for DNAJC9 (Supplementary Fig. 6d now with 3 experiments instead of 2) and we now provide a rescue experiment also for MCM2 showing that re-expression of wild-type MCM2 restores at least partially new H3.3 deposition at UV sites (Suppl Fig. 7f).

Second, as also pointed out by the authors, based on the current literature a number of mechanisms can be invoked to explain how DNAJC9 and MCM2 accumulate at sites of DNA damage to promote histone incorporation. These could be direct binding to histone H3-H4 (histones evicted from chromatin could become 'visible' to these chaperones?); interaction of MCM2 with other CMG components; regulation of 'damaged' chromatin binding by the J domain of DNAJC9. Importantly, point mutants that disrupt these interactions have all been described. The contribution of these mechanisms could be tested in a rescue system, and would transform our understanding of how DNAJC9 and MCM2 function at DNA repair sites.

Through functional rescue experiments, we have tested the impact of DNAJC9 histone binding mutant on the recruitment of this chaperone to sites of UV damage repair and on the resulting repair-coupled histone deposition. We initially planned to test both a histone binding mutant of DNAJC9 and a mutant in the J domain, which abolishes the interaction with Hsp70 family members (PMID: 33857403) but we could not achieve stable expression of the J domain mutant. We observed that expression of wild type DNAJC9 rescued, at least in part, new H3.3 deposition at UV damage sites in DNAJC9-knocked down cells, which was not observed upon expression of a histone binding mutant form of DNAJC9 although both wild type and mutant DNAJC9 were similarly

recruited to UV damage sites (new Fig. 4d). These experiments establish that DNAJC9, through its histone binding domain, promotes the deposition of newly synthesized histones at UV damage sites.

As for MCM2, the H3-H4 histone binding mutant (Y81A, Y90A) is not a perfect separation of function mutant because all MCM2 histone binding mutants also affect the assembly of the CMG replicative helicase complex to some extent (PMID: 26167883). Moreover, stable expression of the MCM2 histone binding mutant was highly cytotoxic, preventing functional rescue experiments with this construct. Note that while the whole MCM hexamer is captured at sites of UV damage repair, other CMG components like Cdc45 and GINS are not (Supplementary Fig. 3c), which indicates that MCM2 likely operates independently of the CMG complex at repair sites (now discussed on p. 17).

To further characterize MCM2 and DNAJC9 function in histone dynamics at repair sites, we ruled out the hypothesis that MCM2 and DNAJC9 contribution to old H3.3 recovery may be an indirect consequence of these chaperones stimulating new H3.3 deposition. Indeed, depletion of the H3.3 chaperone HIRA, known to deposit new H3.3 histones at sites of UV damage repair (PMID: 24074863), did not impair old H3.3 recovery (Supplementary Fig. 7g).

Other points:

Line 116: Figure S1E only shows XPB recruitment, not 'UV damage dependent factors', so the text should be amended accordingly.

We have rephrased this part (p. 6)

Fig. 2D is not very informative, particularly the 'reference number' for the gene ontology terms. Can this panel be reshaped?

We have removed the GO term reference numbers from the representation of the gene ontology analysis in Fig. 2d and included instead additional broad terms for the most enriched GO terms.

How do the authors explain the difference in proteins retrieved by aniFOUND as compared to IPOND-R (Fig. 3A)? adding a short explanation in the text would be helpful for readers to compare the two techniques. And how is the overlap between IPOND-R and IPOND (untreated conditions)?

The modest overlap with the aniFOUND dataset can be explained by the different cell types (U2OS osteosarcoma cells for IPOND-R vs. 1BR3 skin fibroblasts for aniFOUND) and most likely by the different time points post UV (1 h for IPOND-R vs. 4 h for aniFOUND) as early repair responses are not captured 4 h post irradiation (now explained on p. 8).

We have also compared the IPOND-R mass spectrometry dataset (1 h-specific hits in U2OS cells) to the proteins identified on nascent replicated strands in undamaged conditions by IPOND combined with mass spectrometry (593 proteins across 4 human cell lines HEK293T, HCT-116, HeLa and RPE-1 (PMID: 31553917) and 522 proteins in human TIG-3 cells (PMID: 36680776). This analysis revealed several shared replication-associated factors including RFC1-5, PCNA, MCM2-7 but also highlighted important differences between the proteomes at replication forks and at sites of UV

damage repair with replication-associated factors that are not retrieved at sites of UV damage repair such as Cdc45, Claspin, MCM10, TONSL (Supplementary Fig.3c).

This could be due to their low relative abundance and as such may not be detectable depending on yield and coverage of the interactome.

In Figure 4, the pattern of H3.1 incorporation into chromatin is used to define whether cells are in S phase or not. However, given that loss of DNAJC9 decreases new H3.1-SNAP incorporation and the percentage of S-phase cells (Fig. S5G-I), how reliable is the observed H3.1 pattern? Can the authors test whether DNAJC9-depleted cells still consistently show the expected 'S-phase' (early, mid, late) patterns for H3.1-SNAP, p150 and/or EdU? This would validate the use of H3.1-SNAP patterns as a marker of S-phase in Fig. 4.

We can easily discriminate between cells in and outside S phase based on new H3.1 nuclear levels even in DNAJC9-knocked down cells because the levels of new H3.1 are always significantly higher in S phase cells (Supplementary Fig. 6a, S phase cells were identified based on CAF-1 p60, which is unchanged upon DNAJC9 knockdown).

Fig. 4H: Given that DNACJ9 and HIRA depletion leads to lower H3.3-SNAP incorporation, the H3.3-SNAP images seem not very representative. Has the contrast been changed differently between samples to highlight the decrease in H3.3-SNAP accumulation specifically at UVC sites? if so, this should be stated in the legend. The image settings are kept the same between samples. We now show more representative pictures that better reflect the results of the quantification presented on the graph (Fig. 4i).

Reviewer #2:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3:

The paper by Plessier et al, describes a new proteomic method to isolate factors accumulating on newly synthesized DNA after UV DNA damage, named IPOND-R. After validation of the method, the authors focus on analyzing the effect of depletion of DNAJC9 on the dynamics of new and old histones at DNA damage sites. The authors find that DNAJC9 controls the dynamics of both histone populations, but does not affect DNA damage repair per se (i.e. recruitment of repair proteins, or survival after DNA damage). The authors conclude by suggesting an interplay between MCM2 and DNAJC9 to control chromatin dynamics at UV damage sites. The paper contains high quality analyses of the effect of DNAJC9 depletion, and also provides a well validated new approach to study the proteome of DNA damage repair sites. In general, the findings are interesting and may attract attention from several research communities involved in DNA repair and chromatin biology. However, there are few concerns that need to be addressed before publication.

The most critical issue is that it is not clear that the effects described are direct and related to DNA damage repair, rather than indirect effects from the cells going through S-phase without critical histone chaperones. DNAJC9, MCM2 as well as CAF-1 and HIRA are critical histone chaperones and they will alter chromatin dynamics independently of DNA damage, as previously shown. Because the authors do not see an effect of DNAJC9 on DNA repair or survival after damage, it could be that the authors are simply measuring changes in chromatin dynamics that are not dependent on DNA damage, but related to global chromatin alterations. This is due in part to the experimental setup of the assay: the use of siRNA and the conditions used to inhibit DNA replication. Controls should be added to address this concern.

1) The authors run many important controls to validate the IPOND-R approach to minimize the contribution of DNA replication in the dataset. However, treatment of cells with HU and Ara-C to inhibit replicative synthesis also stresses these cells significantly. I wonder if the authors could use synchronization of the cells in G1/S by Palbociclib (which is known to not induce stress) to conclusively demonstrate the power of the method. Running IPOND-R and the follow up (key) experiments in Palbociclib arrested cells will provide important confirmation that DNA replication and S-phase mechanisms are not at play in the chromatin dynamics presented here.

The combination of HU and ara-C indeed triggers genotoxic stress. To address this valid concern, we used instead the cdk4/6 inhibitor Palbociclib to arrest cells in G1. This treatment was applied to RPE-1 cells because U2OS cells are resistant due to high cdk4 expression. We confirmed by EdU staining the complete depletion of replicating cells upon Palbociclib treatment while repair synthesis remained unaffected. However, we noticed that Palbociclib treatment hindered the UV-induced stress response as evidenced by a lack of increase of γ H2A.X signal between non-irradiated and UV-irradiated cells, preventing us from running successful IPOND-R experiments in Palbociclib-treated cells (no enrichment of material in the +UV capture, see Figure for Reviewer below). Nevertheless, we could monitor repair-coupled histone dynamics, such as new H3.3 deposition, in cells arrested in G1 by Palbociclib treatment. Similar to what observed in cycling cells, DNAJC9 and MCM2 knock-down in G1-arrested cells impaired new H3.3 deposition at sites of UV damage repair (Supplementary Fig. 7e),

arguing that the function of these chaperones in histone deposition coupled to repair does not require passage through S phase. Furthermore, DNAJC9 and MCM2 recruitment to UV damaged chromatin was observed outside of S phase (Supplementary Fig. 4b), indicating that they are replication-independent.

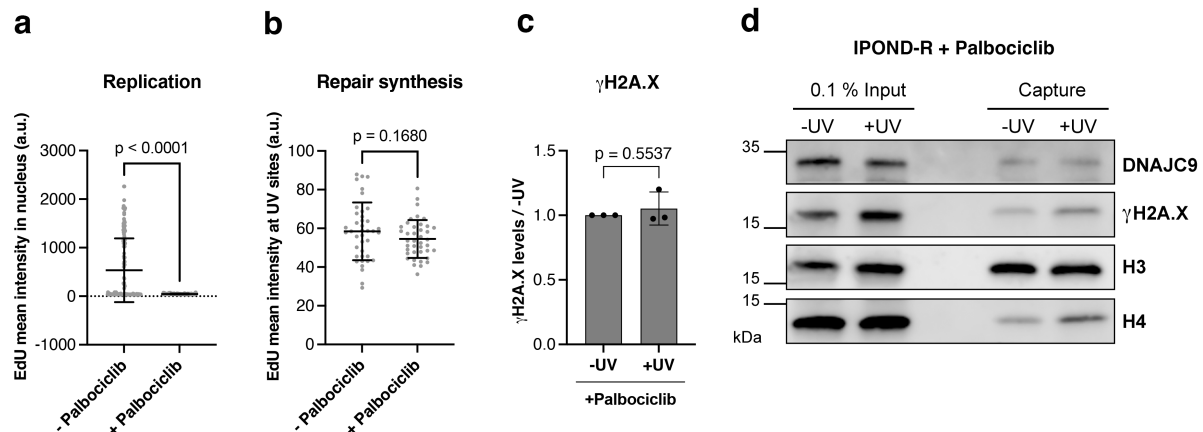


Figure for Reviewer: Set up of IPOND-R in cells arrested in G1 by Palbociclib treatment

(a) Inhibition of replication-coupled DNA synthesis measured by EdU incorporation (pulse of 10 min) in RPE-1 cells treated for 24 h with 500 nM Palbociclib.

(b) Effect of Palbociclib pre-treatment on repair-coupled DNA synthesis measured by EdU incorporation (pulse of 1 h) at sites of local UV irradiation (marked by CPD immunostaining) in RPE-1 cells. S phase cells present in the non-treated population were identified by pan-nuclear EdU staining and excluded from the analysis.

(c) γ H2A.X levels analyzed by western blot in total extracts from RPE-1 cells treated for 24 h with 500 nM Palbociclib and exposed or not to 10 J/m² global UV irradiation. Cells were harvested 1 h post irradiation.

(d) Western blot analysis of proteins captured by IPOND-R 1 h post UV irradiation in RPE-1 cells arrested in G1 by 24 h treatment with 500 nM Palbociclib. -UV is used as negative control.

Dot plots: mean $\pm$ SD. Statistical significance is calculated with a two-sided Student's t-test with Welch's correction.

2) As the authors deplete their proteins with siRNA, the cells are cycling in the absence of the target proteins for at least one full cell cycle. As the authors are targeting critical histone chaperones (DNAJC9, MCM2, CAF-1 and HIRA), it is expected that chromatin is already affected at the time of DNA damage induction. The authors should find ways to circumvent this experimental limitation (i.e. use rapid degron-based degradation or test their key findings in G1 arrested cells).

As explained above, we validated our findings in G1-arrested RPE-1 cells by palbociclib treatment and thus confirmed the contribution of DNAJC9 and MCM2 to repair-coupled histone deposition, independently of cell passage through S phase (Supplementary Fig. 7e).

3) In line 228-229 it is stated that the quantification of Figure 4B focuses on non S-phase cells. It is not clear to this reviewer how one can reliably distinguish S and non S-phase cells from new H3.1 distribution (I can only imagine a difference between early and late S-phase). I expect a difference could be in the total amount of new H3.1 (should be significantly higher in S-phase cells), but not clear how the distribution can alone explain S vs non-S phase cells. At any rate, the authors should include a quantification for S-phase cells to test if there is a difference. Could the authors use

EdU to distinguish various populations otherwise? Or synchronize with Palbociclib as suggested above?

We can easily discriminate between cells in and outside S phase based on new H3.1 nuclear levels even in DNAJC9-knocked down cells because the levels of new H3.1 are always significantly higher in S phase cells (Supplementary Fig. 6a, S phase cells were identified based on CAF-1 p60, which is unchanged upon DNAJC9 knockdown).

4) The involvement of MCM2 is also unusual, without evoking the passage of DNA replication forks. The authors do not really mention this, but this needs to be addressed/discussed. How can they envision MCM2 working at these sites, or together with DNAJC9, outside of DNA replication? Hypotheses need to be tested to clarify their data, which is very preliminary in the current form.

MCM2 is enriched in the IPOND-R dataset together with the other MCM subunits (MCM3 to 7) showing that the whole MCM hexamer is present at sites of UV damage repair like at replication forks. However, the observed recruitment of MCM2 to sites of UV damage repair does not require the passage of replication forks because it is detected outside of S phase (new Supplementary Fig. 4b), highlighting a function of MCM2 that is disconnected from DNA replication. In line with this, we do not retrieve other components of the CMG replicative helicase complex at repair sites like Cdc45 and GINS subunits (Supplementary Fig. 3c, Supplementary Table 1), which indicates that MCM2 likely operates independently of the replicative helicase complex at repair sites. Notably, an abundant pool of MCM proteins is not engaged with active replisomes in human cells (PMID: 36241664) and those could fulfill replication-independent roles (now discussed on p.17-18).

5) In Figure 1D, the authors should probe for DNA replication proteins to show that they are not pulled down (e.g. Cdc45, GINS, or fork protection complex components). The comparison between IPOND-R and IPOND data sets (see point 6 below) reveals important differences between the proteomes at replication forks and at sites of UV damage repair. Some replication-associated proteins are not captured at repair sites including Claspin, Cdc45, MCM10, TONSL (Supplementary Fig. 3c). We confirmed by western blot that the replication-associated protein Cdc45 was not enriched in the +UV capture (Supplementary Fig. 3d).

6) The authors should also show the overlap between IPOND-R and previously reported IPOND hits (in Figure 2), to show how many of the fork proteins are present in the data.

We have compared the IPOND-R mass spectrometry dataset (1 h-specific hits in U2OS cells) to the proteins identified on nascent replicated strands in undamaged conditions by IPOND-mass spectrometry (593 proteins across 4 human cell lines HEK293T, HCT-116, HeLa and RPE-1 (PMID: 31553917) and 522 proteins in human TIG-3 cells (PMID: 36680776). This analysis revealed several shared replication-associated factors including RFC1-5, PCNA, MCM2-7 but also highlighted important differences between the proteomes at replication forks and at sites of UV damage repair with replication-associated factors that are not retrieved at sites of UV damage repair such as Claspin, Cdc45, MCM10, TONSL (Supplementary Fig.3c)

There is an instance of “data not shown” in the discussion (lines 339-340). These data should be shown, or the statement needs to be removed.

We now show that DNAJC9 is loosely bound to UV-damaged chromatin, as it is mostly solubilized by detergents, in contrast to CAF-1 and HIRA (Supplementary Fig. 6n).

Reviewer #4:

The study investigates chromatin dynamics during UV damage repair, focusing on identifying proteins that regulate histone deposition at damaged sites. The authors modified the IPOND proteomic method, naming it IPOND-R, to analyze protein dynamics specific to repair sites. Through this approach, they identified DNAJC9 and MCM2, which had not previously been linked to UV-induced damage. DNAJC9 is suggested to facilitate the deposition of new histones and recovery of parental histones, while MCM2 is proposed to collaborate in these processes to maintain chromatin integrity. This finding adds to the understanding of the dynamic regulation of chromatin following DNA damage. However, several issues undermine the study's conclusions. Many results lack appropriate controls, and the experimental descriptions are insufficiently detailed, making the findings difficult to interpret and raising concerns about their reliability. Furthermore, the logical connections between key results are unclear. These shortcomings lead to ambiguous conclusions, and some interpretations appear to extend beyond what the data can support.

Major:

1. Based on the proposed model of chromatin repair (Figure 6F), it appears that DNA breaks are repaired first, followed by histone deposition to restore chromatin structure. However, the authors did not assess when DNA repair is complete. Notably, the persistence of γ H2AX after 24 hours suggests that repair may not be fully resolved, and DNAJC9 is no longer present at the damage sites at this time point. Furthermore, while DNAJC9 is transiently present at UVC damage sites, the authors report no evidence of its involvement in DNA repair. These findings raise important questions about the proposed model and the role of DNAJC9 at DNA damage sites. To clarify these points and strengthen the model, the authors should provide additional details, including:

- Evidence indicating when DNA repair is complete.
- Whether the loss of DNAJC9 affects transcription recovery near break sites following UVC damage.

Addressing these points will help establish a clearer connection between DNA repair, histone deposition, and the function of DNAJC9 in the context of DNA damage.

Some histone deposition arises before DNA repair completion as shown for new H3.3 deposition, which is coupled to early repair steps, namely UV damage detection (PMID: 24074863). To better illustrate this point, we now indicate on the model in Fig. 6f “New histone deposition coupled to DNA repair”. Therefore, the presence of a histone chaperone on UV-damaged chromatin before repair completion does not preclude an involvement of this chaperone in chromatin restoration coupled to DNA repair.

Evidence for the completion of UV damage repair around 24 h post UV is two-fold: (1) The kinetics of UV lesion removal (Supplementary Fig. 1f-g) shows that 6,4-photoproducts are repaired fast (within 2 h) and the CPDs are removed in 24-30 h. (2) UV damage repair factors are no longer captured by IPOND-R 24 h post UV indicating that most repair is complete by that time. The persistence of some γ H2A.X signal 24 h post UV (Fig. 3d) is not a direct readout of the presence of DNA damage. It shows that DNA damage signaling is not yet turned off, which occurs after repair completion.

To further characterize the function of DNAJC9 in UV-damaged chromatin, we investigated the contribution of this histone chaperone to transcription recovery following UVC damage by labeling nascent transcripts with ethynyl-uridine. Knocking down DNAJC9 did not impair transcription recovery at UV damage sites in contrast to knocking down the transcription-coupled repair factor CSB (Supplementary Fig. 5f). These results are consistent with a previous multiomic analysis of the UV damage response (PMID: 27184836) where DNAJC9 did not stand out as a factor regulating nascent transcription post UV, and rule out a function of DNAJC9 in transcription-coupled repair.

2. The authors treated cells with 10 mM HU and 1 mM AraC to inhibit DNA replication, raising several concerns:

- While AraC can inhibit DNA replication, it is also incorporated into repair sites, potentially causing repair defects. This issue is not addressed in the manuscript, undermining the validity of the experimental approach.
- The use of 1 mM AraC is an extremely high concentration for dividing cells, especially since concentrations as low as 40 μ M can be toxic to some post-mitotic cells. The observation that cells accumulate in the G1 phase even 24 hours post-treatment with DNA synthesis inhibitors suggests that the inhibitors themselves may have induced significant cellular stress.

Given these concerns, we recommend replacing HU+AraC with other inhibitors, such as G1 arrest inhibitors (eg. Palbociclib), which would allow the cells to arrest in G1 phase and prevent EdU incorporation during DNA replication. This adjustment would enhance the reliability of the approach and reduce potential confounding effects.

Although we verified that the combination HU+Ara-C did not cause UV damage repair defects (Supplementary Fig. 1e-g), we acknowledge that such treatment can trigger genotoxic stress (Supplementary Fig. 1a-c). To address this valid concern, also raised by Reviewer #3, we used the cdk4/6 inhibitor Palbociclib to arrest cells in G1. This treatment was applied to RPE-1 cells because U2OS cells are resistant due to high cdk4 expression. We confirmed by EdU staining the complete depletion of replicating cells upon Palbociclib treatment while repair synthesis remained unaffected. However, we noticed that Palbociclib treatment hindered the UV-induced stress response, as evidenced a lack of increase of γ H2A.X signal between non-irradiated and UV-irradiated cells, preventing us from running successful IPOND-R experiments in Palbociclib-treated cells (no enrichment of material in the +UV capture, see Figure for Reviewer below). Nevertheless, we could monitor repair-coupled histone dynamics, such as new H3.3 deposition, in cells arrested in G1 by Palbociclib treatment. Similar to what observed in cycling cells, DNAJC9 and MCM2 knock-down in G1-arrested cells impaired new H3.3 deposition at sites of UV damage repair (Supplementary Fig. 7e), arguing that the function of these chaperones in histone deposition coupled to repair does not require passage through S phase.

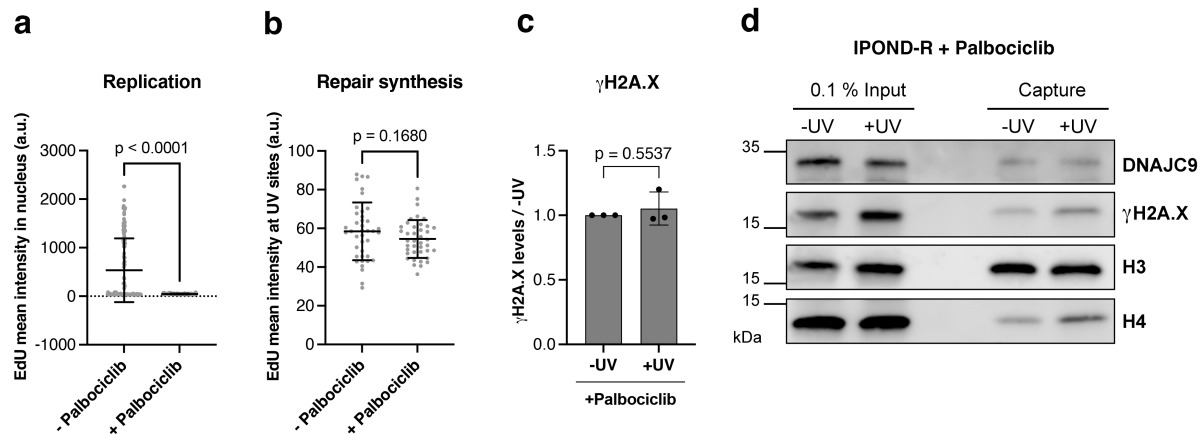


Figure for Reviewer: Set up of IPOND-R in cells arrested in G1 by Palbociclib treatment

(a) Inhibition of replication-coupled DNA synthesis measured by EdU incorporation (pulse of 10 min) in RPE-1 cells treated for 24 h with 500 nM Palbociclib.

(b) Effect of Palbociclib pre-treatment on repair-coupled DNA synthesis measured by EdU incorporation (pulse of 1 h) at sites of local UV irradiation (marked by CPD immunostaining) in RPE-1 cells. S phase cells present in the non-treated population were identified by pan-nuclear EdU staining and excluded from the analysis.

(c) γ H2A.X levels analyzed by western blot in total extracts from RPE-1 cells treated for 24 h with 500 nM Palbociclib and exposed or not to 10 J/m² global UV irradiation. Cells were harvested 1 h post irradiation.

(d) Western blot analysis of proteins captured by IPOND-R 1 h post UV irradiation in RPE-1 cells arrested in G1 by 24 h treatment with 500 nM Palbociclib. -UV is used as negative control.

Dot plots: mean $\pm$ SD. Statistical significance is calculated with a two-sided Student's t-test with Welch's correction.

3. In Figure 5B, siXPG should prevent UV lesion removal, thereby halting DNA repair. However, the data still show a recovery of old H3.3, which is confusing. If DNA repair is blocked, it would be expected to also impact histone deposition. The authors should clarify how histone deposition occurs independently of DNA repair in this context and provide additional insights into DNAJC9's function.

In Fig. 5b, siXPG is indeed used as a positive control because XPG depletion prevents UV lesion removal thus impacting old histone recovery. XPG knock-down still allows some old histone recovery because the siRNA-mediated knock-down does not fully remove the protein thus repair is not 100% abrogated (as now stated on p. 13-14) but old histone recovery is significantly impaired in siXPG compared to the siLuc control condition ($p < 0.0001$).

Minor:

1. To identify proteins that transiently bind to chromatin during repair, the authors compared IPOND-R results at 1-hour and 24-hour post UV time points. However, this approach does not rule out the possibility that cells with the most severe damage at 1 hour post-UV may not survive until the 24-hour time point, thus create bias. To address these concerns, the authors should perform EdU pull-down sequencing to confirm that the EdU-incorporated sites at 24 hours are comparable in abundance and distribution to those at 1 hour post-UV treatment.

We show that the levels of incorporated EdU in cell nuclei are not lower at 24 h compared to 1 h post UV (Supplementary Fig. 2b), arguing against the death of UV-

damaged, EdU-labeled cells between 1 h and 24 h post irradiation (now stated on p.6). Furthermore, the amount of UV damage is quite homogeneous in a cell population as shown by CPD staining immediately after global UVC irradiation (new Supplementary Fig. 2c) so there is not a large proportion of cells with more severe damage and it does not introduce any bias in the analysis.

2. The analysis in Figures 4 and 5 presents data at 20 minutes, 2 hours, and 8 hours post-UVC exposure without providing any explanation for this shift, despite earlier data focusing on 1 hour post-UVC. This raises questions about whether DNAJC9 remains present at UV damage sites at these time points in the control. To address these issues, the authors should provide a clear rationale for the time-point selection.

Twenty minutes post UV was previously determined as the best time point to visualize HIRA accumulation at UV sites, 2 h is the best time point to visualize CAF-1 and new H3 deposition at repair sites (PMID: 24074863) and 8 h is suited to visualize old histone recovery (PMID: 27642047). In addition to the 1 h and 8 h time points (Fig. 3e-f and 6a), we now also show DNAJC9 and MCM2 enrichment at sites of UV damage repair 20 min and 2 h following local UVC irradiation (Supplementary Fig. 4a).

3. The IF results for new H3.3-SNAP in siDNAJC9#3 in Figures 4C and 4H display inconsistent deposition patterns (completely absent in one instance and still present in another), which raises concerns about data reliability. The authors should use figures displaying multiple cells instead of single cells to ensure greater consistency and reliability in the presentation of the data.

We now show more representative pictures in Fig. 4i that better reflect the results of the quantification presented on the graph. We believe that quantifying the signal in at least 150 cells scored in 3 independent experiments is the most reliable way of presenting the data.

4. There is no description of how the survival assay was performed. It is unclear whether the UVC dose of 10 J/m² causes 85% cell death within 24 hours, 48 hours, or over the course of a week, which creates confusion and raises questions about the experimental conditions.

The description of the survival assay was present in the method section under the caption “Colony forming assays”, which we have now rephrased as “Cell survival assays” for clarity. In these assays, cell colonies are counted after 12 days of growth following UV irradiation. As previously stated, there is hardly any detectable cell death within 24 h post UV.

In light of these concerns, the paper's conclusions about DNAJC9's role in chromatin repair appear premature and not fully supported by the data presented. A substantial revision addressing these issues would be necessary to strengthen the study's findings and interpretations.

Reviewer #5:

Please see comments below in red. Some are major concerns which are easily addressed, yet authors have not attempted to address them. Specifically, my main concerns are surrounding the significance of the data presented in Figure 5. I have detailed steps to address my concerns below, and these should be addressed prior to acceptance for publication.

In addition to the steps below, I noted Sandra Piquet is missing from the author list in the manuscript file.

Plessier et al presents an innovative proteomic approach to capture chromatin in the process of UVC irradiation repair based on the IPOND protocol (IPOND-R). This protocol relies on the complete inhibition of DNA replication such that EdU is only incorporated during UV-damage coupled DNA repair processes. As can be seen in Figure 1B, the inhibition of DNA replication with HU and Ara-C combined is highly effective. Furthermore, the post UVC repair EdU signal is similar in non S-phase cells when not treated with these inhibitors (Figure 1C). These are important controls since small amounts of DNA replication could significantly skew the interpretation of the resulting proteomic analyses from these experiments. Whilst the proteomic datasets have merit, there should be a more detailed comparison and benchmarking of them compared to classical iPOND experiments. For example, by comparing them to the datasets in Sirbu et al JBC VOL. 288, NO. 44, pp. 31458 –3146 (2013). This would help to highlight the differences to factors identified at the replication fork to those enriched at sites of UV damage repair.

As suggested, we have compared the IPOND-R mass spectrometry dataset (1 h-specific hits in U2OS cells) to the proteins identified on nascent replicated strands in undamaged conditions by IPOND-mass spectrometry (593 proteins across 4 human cell lines HEK293T, HCT-116, HeLa and RPE-1 (PMID: 31553917) and 522 proteins in human TIG-3 cells (PMID: 36680776). This analysis revealed several shared replication-associated factors including RFC1-5, PCNA, MCM2-7) but also highlighted important differences between the proteomes at replication forks and at sites of UV damage repair with replication-associated factors that are not retrieved at sites of UV damage repair such as Cdc45, Claspin, MCM10, TONSL (Supplementary Fig.3c).

Thank you for including this analysis in the revised manuscript. Please include the source data for the Venn Diagram in the supplemental excel file since it would be useful for the field to have the lists of proteins identified in each section of the Venn Diagrams depicted in Supplementary Figure 3b-c.

Beyond this, I was not sufficiently convinced by the follow-up studies whereby DNAJC9 was selected as the key factor in this process despite being close to the cut-off for statistical significance in the proteomic datasets. The most convincing aspect of this was that depletion of DNAJC9 causes a loss of new SNAP tagged H3.3/H3.1. In the UV irradiated experiments, the signal of DNAJC9 and MCM2 at irradiated sites was not convincing, no easily observable foci are formed. Furthermore, since this could

also be explained by bleed through from the GFP channel into the red channel, and I was not able to find evidence that this has been correctly controlled for, this calls into doubt the validity of conclusions made based on this approach – thus at this stage it is unclear of the significance of many findings.

DNAJC9 was chosen with MCM2 because those were the only two histone chaperones in the IPOND-R capture that had not previously been involved in the UV damage response. Even if close to the cut-off for statistical significance, DNAJC9 recruitment was confirmed at UV sites by imaging approaches (Fig. 3e-f). We acknowledge that the observed enrichment of DNAJC9 at UV sites is rather mild and this is likely due to the fact that DNAJC9 is loosely bound to chromatin contrary to CAF-1 and HIRA chaperones (Supplementary Fig. 6n). MCM2 recruitment is more readily detectable at late time points post irradiation (Fig. 6a) and we verify that the observed histone chaperone signal enrichment at UV damage sites does not result from a bleed-through from the UV damage channel (new Supplementary Fig. 7a). To consolidate our findings, we examine more thoroughly the recruitment of DNAJC9 and MCM2 to sites of UV damage repair at different time points following damage infliction (Fig. 3e-f, Supplementary Fig. 4a, Fig. 6a-b) and we demonstrate that their recruitment to UV sites occurs outside of S phase (Supplementary Fig. 4b). DNAJC9 recruitment to UV damage sites 8h post irradiation was not very convincing and we thus decided to remove the data from the manuscript.

Functionally, our follow-up studies demonstrate that DNAJC9 affects histone H3 dynamics at repair sites, which we confirmed and further characterized through functional rescue experiments (Fig. 4d). These experiments establish that DNAJC9, through its histone binding domain, promotes the deposition of newly synthesized H3 variants at UV damage sites. In addition, we have run additional control experiments showing that the function of DNAJC9 in new histone deposition at repair sites does not require the passage of cells through S phase (Supplementary Fig. 7e).

The data in figure 4d shows quite clearly that exogenous DNAJC9 expression does rescue new H3.3 levels at UVC DNA repair sites and this is not observed in the 4A histone binding mutant. This is an excellent addition to the manuscript.

Furthermore, the statistical handling of data is questionable. Whilst microscopy experiments have been performed with three biological replicates, paired t-tests appear to use all data points from these experiments rather than the three means of the biological replicates. This is a very confusing way to present the data and makes it difficult to assess how robust the conclusions made are. For example, are the changes in Figure 5 really strong enough to conclude that DNAJC9 has a role in old histone recovery/histone recycling?

Together, whilst I believe this is a promising story, it is not yet sufficiently developed for publication in Nature Comms.

We run the statistical analyses on all data points and not on the means of the biological replicates in conditions of local UV irradiation because in this case each nucleus receives a different UV dose, which depends on the pore distribution in the micropore filters used for local UVC irradiation (mostly 1 or 2 and up to 5 UV spots per nucleus, PMID: 39820273), so each nucleus should be considered independently. This is now stated in the method section (p. 35).

On page 6 the authors state that “Damage induction is quite homogenous in a cell population (Supplementary Fig. 2c) so this does not introduce any bias in subsequent analyses.” This is at odds with the statistical handling of the data in Figure 5 and 6, where all data points across three biological replicates are used instead of the three biological replicate means during statistical testing. In addition, since the above critique would have been an easy point to rebut by simply recalculating P values based on the three biological replicate means, does this mean that maintaining the current statistical representation is the only way to maintain the significance of this observation?

Figure 4d should have indication of the UVC treatment in the figure (time post local UVC), similar to 4b.

Even if modest, the defects observed upon knockdown of DNAJC9 are comparable to the defects observed upon depletion of the HIRA and CAF-1 chaperones for new histone deposition (Fig. 4h-i) and comparable to the defects observed upon depletion of the late repair factor XPG for old histone recovery (Fig. 5b). Furthermore, the impaired recovery of old histones observed upon DNAJC9 depletion cannot be attributed to defective UV damage repair because DNAJC9-knocked down cells are fully proficient for the repair of UV lesions (Supplementary Fig. 5). This is now explained on p. 14 of our revised manuscript.

In addition, why does siLuc in Figure 5b promote over-recovery of the TMR mean intensity at UVC sites/nucleus (the mean is >1)? In contrast with siDNAJC9 and siXPG the TMR mean intensity at UVC sites/nucleus is approx. $=1$, wouldn't this be consistent with full signal recovery? Therefore, wouldn't this data argue that DNAJC9 and XPG have no role in old histone recovery after UV damage. I therefore question the interpretation of the data here, and question whether this analysis is actually worthy of inclusion in the publication. I would consider the analysis in Supplementary Figure 7f as more pertinent to the story, this panel demonstrates the role of MCM2 in new H3.3 deposition at UVC sites, a novel discovery that should be given more prominence.

To further strengthen the role of DNAJC9 and MCM2 in old histone recovery, we ruled out the hypothesis that MCM2 and DNAJC9 contribution to old H3.3 recovery may be an indirect consequence of these chaperones stimulating new H3.3 deposition. Indeed, depletion of the H3.3 chaperone HIRA, known to deposit new H3.3 histones at sites of UV damage repair (PMID: 24074863), does not impair old H3.3 recovery (Supplementary Fig. 7g).

This is an independent experiment, as such an experiment that included siRNA depletion of DNAJC9 and MCM2 would be more convincing. E.g. in the same experiment comparing.

- 1) siControl
- 2) siDNAJC9
- 3) siMCM2
- 4) siHIRA
- 5) siDNAJC9+siMCM2
- 6) siDNAJC9+siHIRA
- 7) siMCM2+siHIRA

One final point is that the conclusion that siDNAJC9 affects the proportion of cells in S phase (Supplementary figure 6h) is based on the proportion of cells that are positive for the histone chaperone Caf1. Can this result be repeated with another more classical readout? E.g. FAC analysis, or using microscopy read-outs e.g. BrdU or EdU?